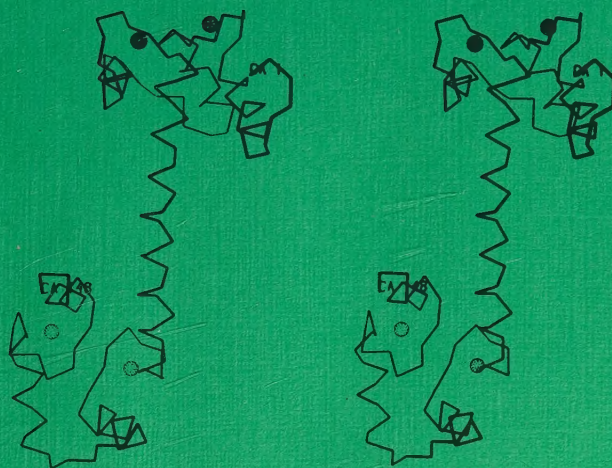

**Calmodulin:
Binding of Cations and Drugs Studied by
NMR and Stopped-Flow Techniques**



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Abstract

Binding of cations and drugs to calmodulin was studied by nuclear magnetic resonance (NMR) and stopped-flow techniques.

NMR studies of the complex formed between calmodulin and various fragments of calmodulin reveal the structural changes between these two proteins. The binding of cations and drugs to calmodulin was studied by stopped-flow techniques. The results show that the binding of cations and drugs to calmodulin is largely independent of the protein structure.

Binding of cations and drugs to calmodulin was studied by stopped-flow techniques. The results show that the four Ca^{2+} binding sites in calmodulin can be grouped into two with higher and two with lower affinity. The difference in affinity is most pronounced at low ionic strength. The binding of drugs to calmodulin was studied by stopped-flow techniques. The results show that the binding of drugs to calmodulin is largely independent of the protein structure.

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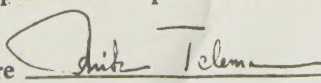
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Abstract Binding of cations and drugs to calmodulin and its proteolytic fragments were studied by nuclear magnetic resonance (NMR) and stopped-flow techniques. ^1H NMR studies of the tryptic fragments comprising the amino- or carboxy-terminal half of calmodulin reveal the great structural similarity between these two proteolytic fragments and the intact protein. Since this was observed for the apo protein as well as the Ca^{2+}-saturated protein, this means that the two halves of the protein are largely independently folded. Studies using ^1H NMR, ^{43}Ca NMR, ^{113}Cd NMR and stopped-flow fluorescence show that the four Ca^{2+} binding sites in calmodulin can be grouped into two with higher and two with lower affinity. The difference is most pronounced at low ionic strength. The strong sites have been identified as sites III and IV in the carboxy-terminal half. Binding of drugs affect all four cation binding sites as inferred from ^1H NMR, ^{113}Cd NMR and stopped-flow fluorescence studies. Trifluoperazine binding to calmodulin and its proteolytic fragments were studied in some detail by ^1H NMR, ^{19}F NMR, ^{113}Cd NMR and stopped-flow fluorescence. These studies reveal the presence of two distinct hydrophobic interaction sites, one located on the amino-terminal and the other on the carboxy-terminal. The strongest trifluoperazine interaction site is located on the carboxy-terminal half. Ca^{2+} dissociation rates and activation parameters for calmodulin and its tryptic fragments were determined by ^{43}Ca NMR and stopped-flow fluorescence. The kinetic properties of the two proteolytic fragments are closely similar to the fast and slowly dissociating sites of native calmodulin, supporting the idea that calmodulin is constructed from two largely independent domains. Correlation times were measured by ^{43}Ca NMR for calcium ions bound to the tryptic fragments.		
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List of Papers

This thesis is based on the following papers (referred to in the text by their Roman numerals) and some unpublished results presented in sections 2.4., 3.2. and 4.

- I. Cadmium-113 Nuclear Magnetic Resonance Studies of Proteolytic Fragments of Calmodulin: Assignment of Strong and Weak Cation Binding Sites
Anita Andersson, Sture Forsén, Eva Thulin and Hans J. Vogel
Biochemistry 22, 2309-2313 (1983).
- II. Metal Ion and Drug Binding to Proteolytic Fragments of Calmodulin: Proteolytic, Cadmium-113, and Proton Nuclear Magnetic Resonance Studies
Eva Thulin, Anita Andersson, Torbjörn Drakenberg, Sture Forsén and Hans J. Vogel
Biochemistry 23, 1862-1870 (1984).
- III. Kinetics of Calcium Dissociation from Calmodulin and Its Tryptic Fragments. A Stopped-Flow Fluorescence Study Using Quin 2 Reveals a Two-Domain Structure
Stephen R. Martin, Anita Andersson Teleman, Peter M. Bayley, Torbjörn Drakenberg and Sture Forsén
Eur. J. Biochem. 151, 543-550 (1985).
- IV. Kinetics of Ca^{2+} Binding to Calmodulin and Its Tryptic Fragments Studied by ^{43}Ca NMR
Anita Teleman, Torbjörn Drakenberg and Sture Forsén
Submitted for publication (1986).
- V. A ^{113}Cd and ^1H NMR Study of the Interaction of Calmodulin with D600, Trifluoperazine and Some Other Hydrophobic Drugs
Anita Andersson, Torbjörn Drakenberg and Sture Forsén
Eur. J. Biochem. 134, 459-465 (1983).
- VI. The Interaction of Various Drugs with Calmodulin as Monitored by ^{113}Cd NMR
Anita Andersson, Torbjörn Drakenberg and Sture Forsén
In "Calmodulin Antagonists and Cellular Physiology" (Hidaka, H. and Hartshorne, D. J., eds) pp 27-44, Academic Press, Orlando (1985).

1. Introduction

This work deals with the binding of cations and drugs to the calcium binding protein calmodulin and its proteolytic fragments with an aim to gaining an understanding of how calmodulin (CaM) can regulate such a wide spectrum of different metabolic processes. Questions of eminent importance to this understanding are the order in which the four Ca^{2+} binding sites are filled and the discrete structural changes that accompany Ca^{2+} binding. The tools used are nuclear magnetic resonance (NMR) and stopped-flow fluorescence. Some aspects on the used techniques are outlined in section 2.

Calmodulin. Since the detection sixteen years ago of a protein factor that stimulated the activity of preparations of brain nucleotide phosphodiesterase [Cheung, 1970; Kakiuchi and Yamazaki, 1970], an enormous number of papers has been published concerning studies of the protein, later called calmodulin. The protein in question is small ($M_r = 16,700$), heat-stable and acidic. CaM consists of a single 148-amino-acid polypeptide chain containing four Ca^{2+} binding sites and is highly conserved throughout evolution [Klee and Vanaman, 1982]. It is found in all eukaryotic organisms so far examined and belongs to a family of structurally related calcium-binding proteins. This class of proteins contains several helix-calcium binding loop-helix regions, often referred to as EF-hands [Kretsinger and Nockolds, 1973]. Upon binding calcium ions the protein undergoes conformational changes that expose sites on the surface that will allow CaM to interact with and modulate the activity of a number of enzymes, ion pumps and other proteins [Klee and Vanaman, 1982]. In this manner CaM's biological function is apparently to mediate the concentration changes of the intracellular messenger Ca^{2+} into a metabolic response.

The three-dimensional structure of CaM was recently reported by Babu et al. [1985]. The structure is remarkable in that it consists of two globular lobes connected by a long α -helix, see cover. A schematic representation is shown in Figure 1.

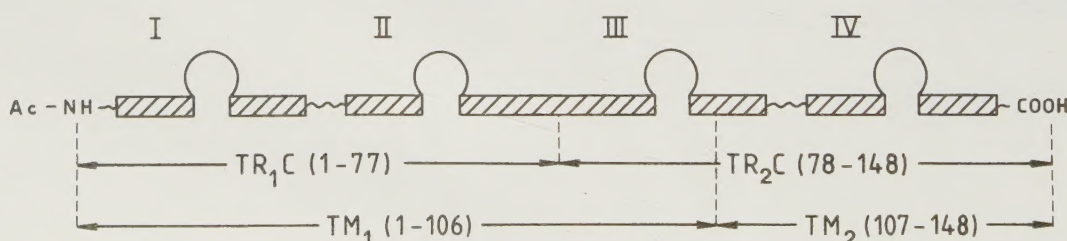


Figure 1. Schematic representation of the structure of calmodulin outlining the four domains, each comprising a calcium binding loop and two flanking helix regions (hatched areas). The proteolytic fragments are also indicated.

2. Experimental Aspects

Most elements of the periodic table have at least one isotope with nuclear magnetic moment. This fact offers several different approaches to study a system by the nuclear magnetic resonance (NMR) technique. In this work, different approaches have been combined to obtain information of a complementary nature. In addition to the NMR method, I have employed the stopped-flow fluorescence technique. The different approaches used are outlined in section 2.1. Other experimental aspects touched upon in this section include exchange rates, the use of $^{113}\text{Cd}^{2+}$ and proteolytic fragments.

2.1. Different Approaches to the Study of Calmodulin

In the work presented in this thesis different techniques have been used to obtain information on binding of cations and drugs to calmodulin. In the study of cation binding to CaM the techniques can be divided into three main types. Firstly, direct study of the bound ion with ^{43}Ca and ^{113}Cd NMR. Secondly, ^1H NMR study of the conformational changes induced by cation binding. Thirdly, stopped-flow fluorescence studies using a Ca^{2+} chelator to measure the dissociation of Ca^{2+} ions from CaM. Similarly the study of drug binding to CaM can be divided into three main types. Firstly, ^1H NMR studies of the conformational changes in the protein induced by drug binding. Secondly, changes in the cation site induced by drug binding, i.e. ^{113}Cd NMR and stopped-flow fluorescence. Thirdly, direct study of the bound drug with ^{19}F NMR.

2.2. Exchange Rates Determined by ^{43}Ca NMR

The ^{43}Ca nucleus possesses a spin quantum number, I , of $7/2$. As for any nucleus with a spin $I > 1/2$, the magnetic relaxation of the nucleus will be dominated by quadrupole relaxation. The resulting ^{43}Ca NMR signal of $^{43}\text{Ca}^{2+}$ ions bound to a protein is usually broad, cf. paper IV. Changes in chemical shifts when more Ca^{2+} is added or the temperature is altered are normally negligible, which makes the relaxation rate the important parameter in ^{43}Ca NMR studies of proteins. The line shape of the observed signal contains information about the dynamics of binding. *Intermediate to fast exchange.* The system is in the intermediate exchange regime when the exchange rate of the calcium ions is of the same order as the relaxation rate of the protein-bound ions. In this regime complicated bandshapes are observed and a total bandshape analysis is necessary to obtain exchange rates. In paper IV Ca^{2+} exchange rates are determined for CaM and its tryptic fragments from the temperature dependence of the ^{43}Ca NMR spectra. A calculation scheme based on theoretical work by Halle and Wennerström [1981] and Westlund and Wennerström [1982] is presented in detail elsewhere [Drakenberg et al., 1983; Drakenberg and Forsén, 1983]. *Slow exchange.* When the exchange rate of the calcium ions is much slower than the intrinsic

relaxation rate(s) the slow exchange regime results. A slow-exchange spectrum is a superposition of the sub-spectra due to each species present, cf. Figure 4 in paper IV. Only an upper limit of the exchange rate can be obtained.

2.3. Cd^{2+} as a Substitution Probe for Ca^{2+}

The use of isotopically enriched chemicals in combination with the Fourier transform technique, developments in probe design and other instrumental improvements have made ^{43}Ca NMR measurements possible for protein solutions at millimolar concentrations. However, resonances for this quadrupolar ion bound to biological macromolecules are usually difficult to observe and resonances of $^{43}\text{Ca}^{2+}$ ions bound to different sites in a protein are rarely resolved [Vogel et al., 1985]. Such is the case for Ca^{2+} bound to CaM. In order to obtain information about the different cation binding sites in CaM an attractive approach is to substitute Ca^{2+} with $^{113}\text{Cd}^{2+}$ or $^{111}\text{Cd}^{2+}$. These spin $I = 1/2$ nuclei give NMR signals that are narrow compared to signals for ^{43}Ca . Moreover, the chemical shift of the ^{113}Cd NMR signal is very sensitive to the nature of the ligands and the coordination symmetry [Cavé et al., 1982; Ellis, P. D., 1983]. Although Ca^{2+} and Cd^{2+} have similar ionic radii, 0.099 and 0.097 nm, respectively, and the same charge, it is not obvious that Cd^{2+} is a good substitution probe for Ca^{2+} . Cd^{2+} may be considered as a valid probe for Ca^{2+} if the following conditions are fulfilled. Firstly, the conformational changes in CaM induced by binding of Cd^{2+} should be the same or very similar to those induced by binding of Ca^{2+} . Secondly, the sequence of cation binding to CaM should be the same for Ca^{2+} and Cd^{2+} . Thirdly, Cd^{2+} bound to the cation sites should result in a CaM molecule which functions biologically. A demanding test of the first and second condition is to study the changes in the ^1H NMR spectra caused by the conformational changes of CaM upon Ca^{2+} or Cd^{2+} titration. As can be seen in Figure 2 only minor differences are observed between the conformations of Ca_nCaM and Cd_nCaM . The third requirement seems to be fulfilled as well since Cd_4CaM activates enzymes like Ca_4CaM does [Chao et al., 1984]. Thus it can be concluded that Cd^{2+} is a reliable probe for Ca^{2+} in this system. The same conclusion has been drawn for the tryptic fragments, see papers I and II.

2.4. Effects of T_1 on Signals in ^{113}Cd NMR Spectra

Measurements of the relative intensities of ^{113}Cd NMR signals from macromolecules with several sites will depend on the longitudinal relaxation time, T_1 , of the different signals unless very long ($\geq 5 T_1$) intervals between rf pulses are employed. This latter approach is usually not very practical for very weak signals requiring a very long accumulation time. In order to assess possible errors involved in NMR signal intensities introduced through the use of short pulse intervals, T_1

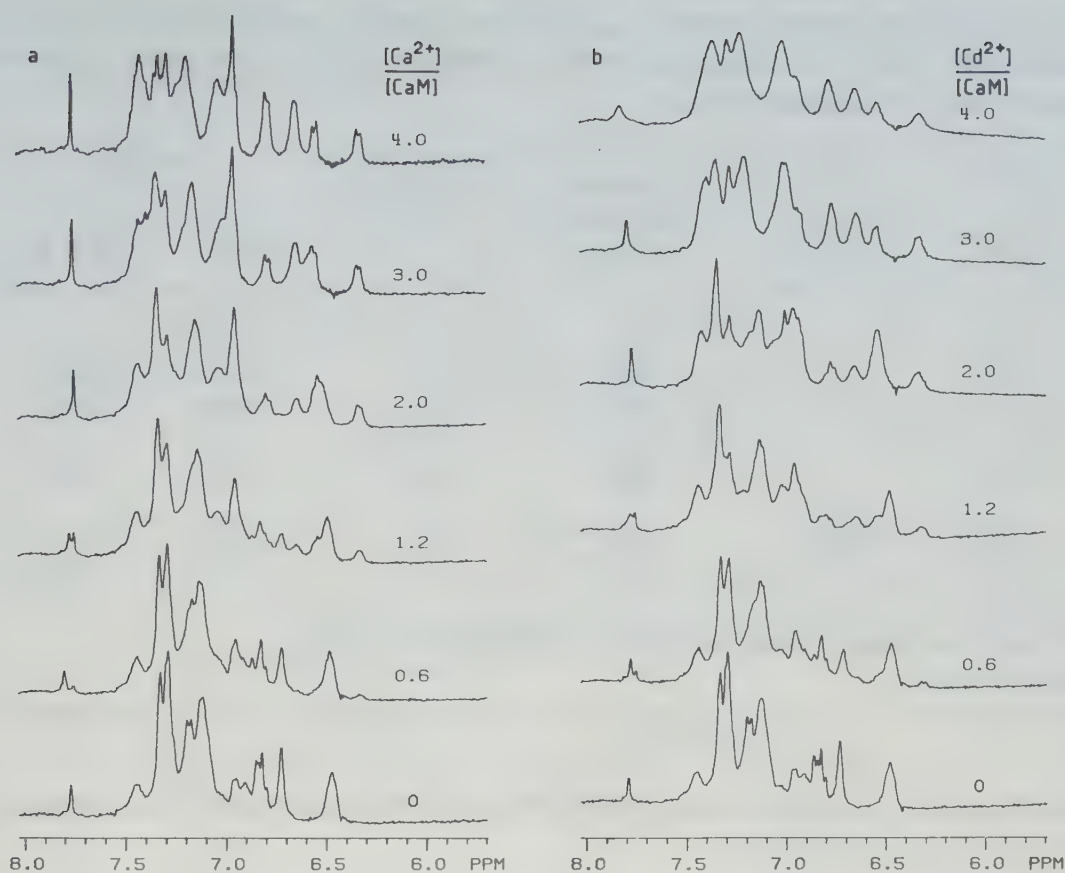


Figure 2. Aromatic part of the 360 MHz proton NMR spectrum of calmodulin (2.0 mM, pH 7.5) (a) titrated with Ca^{2+} and (b) titrated with Cd^{2+} . Note that the series of spectra up to 2 equivalents are in slow exchange and those between 2 and 4 equivalents are in fast exchange. Each spectrum represents the sum of 500 accumulated scans.

values for the signals in ^{113}Cd NMR spectra of Cd^{2+} ions bound to calmodulin were determined by the progressive saturation method [Martin et al., 1980]. The obtained T_1 values, 1.3 and 1.4 s for signals A and B (cf. Figure 4 in paper I), respectively, for Cd^{2+} -saturated bovine testis calmodulin are larger than values reported earlier for bovine brain calmodulin [Forsén et al., 1980]. However, the T_1 values reported in Table 1 are similar to values obtained for structurally related calcium-binding proteins, i.e. parvalbumin [Drakenberg, unpublished results] and troponin-C [Vogel et al., 1983]. From Table 1 it can be seen that the longitudinal relaxation time for signals A and B decreases as the ratio of Cd^{2+} to CaM is increased and approaches the values of Forsén et al. [1980].

The presence of the drug D600 (cf. Table 2) increases the T_1 values for signals A and B considerably, Table 1. At a ratio of D600:CaM of 4:1, the resonances from the two low-affinity cation sites are visible (cf Figure 2 in paper V). The T_1 values for these resonances, 2.3 and 2.7 s

Table 1. Longitudinal relaxation time, T_1 , for the two signals A and B in the ^{113}Cd NMR spectrum of calmodulin. The T_1 values are measured at different Cd^{2+} to CaM ratios, in the absence or presence of drug and at different fields.

$[\text{Cd}^{2+}]$ [CaM]	[D600] [CaM]	Larmor frequency (MHz)	[CaM] (mM)	T_1^a (s)	
				signal A	signal B
2.7	-	56.5	1.7	1.6	1.7
4.0	-	56.5	1.4	1.3	1.4
5.4	-	56.5	1.6	0.80	0.83
4.0	-	80.2	2.0	0.90	0.88
4.0	2.0	56.5	1.7	2.5	2.2
4.0	4.0	56.5	1.6	3.8	2.4

^aThe T_1 values were obtained from a least-square fit to $M_z = M_{\infty}(1 - C\exp(-\tau/T_1))$.

In the ideal case, $C=1$.

for the signals at -113 and -116 ppm, respectively, are of the same order as the values of signals A and B under the same condition.

Measurement of T_1 values at higher magnetic field results in shorter values for both signal A and B, Table 1. Although the relaxation measurements are by no means exhaustive it appears that chemical shift anisotropy can be regarded as a major relaxation mechanism for $^{113}\text{Cd}^{2+}$ ions bound to CaM. The same conclusion has been drawn for $^{113}\text{Cd}^{2+}$ bound to parvalbumin [Vogel et al., 1983].

In order to avoid effects of T_1 on the intensities of the signals, the waiting time between pulses should be at least 5 times longer than the longest T_1 value. In the studied protein system, a long waiting time implies an unreasonable time-consuming experiment. However, the waiting time can be shortened without serious saturation problems if a 45° pulse is used instead of a 90° pulse. This technique has been used in the present study. Caution has all the same to be exercised when interpreting alterations in the intensities of ^{113}Cd NMR signals from CaM as the T_1 values are differing slightly.

Table 2. Chemical structures of drugs.

Drug	Structure
Trifluoperazine	
D600	
W-7	
Felodipine	

2.5. Use of Proteolytic Fragments

Proteolytic fragments of CaM have been of very great help in the assignment of the high- and low-affinity sites in CaM as well as the drug binding sites on CaM. Both thrombic and tryptic fragments have been studied in the present work.

Tryptic fragmentation of Ca^{2+} -saturated CaM takes place mainly at Lys-77 (cf paper II) which is in the middle of the long exposed α -helix that connects the two globular lobes [Babu et al., 1985]. In the crystal structure no contact other than the α -helix is observed between the two lobes, suggesting that the cleavage should leave the two halves of CaM unaffected. In fact, studies using

several different spectroscopic methods show that the spectroscopic properties as well as other properties of CaM are well represented as a sum of the properties of the two tryptic fragments [papers I-IV; Drabikowski et al., 1982; Forsén et al., 1983; Aulabaugh et al., 1984; Dalgarno et al., 1984].

Thrombic digestion of CaM in the presence of EDTA gives two fragments, TM₁ (1-106) and TM₂ (107-148). Cleavage occurs in the carboxy-terminal α -helix of calcium binding domain III. From the data presented in papers I and II, it is obvious that the three-dimensional structure of the carboxy-terminal half is destroyed by the thrombic cleavage.

Thus results obtained with the tryptic (but not the thrombic) fragments can be extrapolated to intact CaM.

3. Cation Binding to Calmodulin

The regulatory effect of calmodulin is intimately linked with its ability to bind Ca²⁺ ions. Questions of interest concern the order and the rate by which the four calcium binding sites are filled with Ca²⁺ ions. The binding sequence of cations to CaM and the kinetics of Ca²⁺ binding to CaM are discussed in this section.

3.1. Binding Sequence of Ca²⁺ Ions to Calmodulin

Ca²⁺ binding to calmodulin can be divided into two distinct stages according to the changes observed in both ¹H and ¹¹³Cd NMR spectra, papers I, II and VI. Stage I: A slow-exchange behaviour is observed up to two equivalents of Ca²⁺ or Cd²⁺ per molecule of CaM. In both ¹H and ¹¹³Cd NMR one set of resonances increases linearly and in ¹H NMR another set of resonances concomitantly decreases. Stage II: A fast-exchange behaviour is observed between 2 and 4 Ca²⁺ or Cd²⁺ per molecule of CaM. In ¹H NMR, resonances are gradually displaced from one chemical shift position to another while in ¹¹³Cd NMR no change is observed in the spectrum of CaM. The ¹¹³Cd NMR signals from the third and fourth cation binding sites are not observed presumably due to exchange broadening.

By comparison of the ¹H and ¹¹³Cd NMR spectra of the proteolytic fragments and CaM, it was possible to assign Ca²⁺ binding domains III and IV as the high-affinity sites and domains I and II as the low-affinity sites, papers I and II. Other researchers have suggested other schemes for the ordered filling of CaM's binding sites, for a discussion see papers I and II. However, the use of trivalent lanthanides (Ln (III)) [Kilhoffer et al., 1980a,b; Wang et al., 1982; Wallace et al., 1982; Krebs and Carafoli, 1982] to probe the calcium binding sites of CaM may be questioned. Although lanthanide ions at low concentrations do support CaM activity [Chao et al., 1984; Klee and

Vanaman, 1982; Wallace et al., 1982], they need not bind to CaM in the same order as Ca^{2+} ions. In fact, results obtained with ^1H NMR to be further discussed in section 3.2., show that the sequence of filling for Lu^{3+} and La^{3+} differs from that for Ca^{2+} and Cd^{2+} .

Upon Cd^{2+} titration the two well resolved ^{113}Cd NMR signals (A and B, cf. Figure 4 in paper I) observed for either CaM or TR_2C increase in intensity nearly in parallel up to a $\text{Cd}^{2+}:\text{CaM}$ (or TR_2C) ratio of 2, papers I and VI. Moreover, in ^1H NMR spectra no resonance is observed for the species CaCaM . Thus, cation binding domains III and IV most likely bind metal ions in a positive cooperative fashion. The same behaviour has been observed for skeletal and cardiac troponin C [Teleman et al., 1983]. In relation to structural features, it is interesting to note that there are hydrogen bond interactions between adjacent Ca^{2+} -binding loops in each half of CaM [Babu et al., 1985]. Moreover, disturbance of the interaction between domains III and IV, which occurs in the thrombic fragments, markedly reduces the affinity of these two sites for Ca^{2+} ions, paper I.

3.2. Lu^{3+} and La^{3+} Binding to Calmodulin

Lanthanide cations have, because of their attractive spectroscopic properties, been used as calcium probes in studies of calcium-binding proteins. In particular, terbium luminescence has been used to examine the binding sequence of Ca^{2+} ions to CaM [Kilhoffer et al., 1980a,b; Wallace et al., 1982; Wang et al., 1982]. However, no evidence has been presented that Ln(III) has the same sequence of filling as Ca^{2+} . In order to test this, the conformational changes upon titration of CaM with Lu^{3+} and La^{3+} were followed by ^1H NMR. The diamagnetic lanthanides Lu^{3+} and La^{3+} were chosen because the paramagnetic properties of the other lanthanides cause undesired alterations in chemical shifts and/or relaxations rates of NMR signals.

In Figure 3 the aromatic regions of ^1H NMR spectra of calmodulin after addition of Lu^{3+} or La^{3+} ions are shown. Some resonances that are well resolved from other resonances were followed during the titrations. These resonances can be divided into three groups. Group I: During the first half of the Lu^{3+} or La^{3+} titration one set of resonances decreases and concomitantly another set increases. For example Asp-64 [Dalgarno et al., 1984] at 5.6 ppm in the metal-free state and at 5.4 ppm in the metal-saturated state. Group II: During the second half of the Ln^{3+} titration a similar slow conformational exchange behaviour is observed but other resonances are involved. For example Tml-115 at 3.13 ppm in the metal-free state and at 3.11 ppm in the metal-saturated state. Group III: Changes of some signals observed during the whole Ln^{3+} titration or at other stages than for group I or II. For example, the signal from His-107 at 7.8 ppm exhibits a two-phase behaviour during the Ln^{3+} titration and the signal from a phenylalanine at 6.45 ppm decreases between 1 and 3 Ln^{3+} per molecule of CaM.

As indicated above a slow conformational exchange (on the proton NMR time scale) is

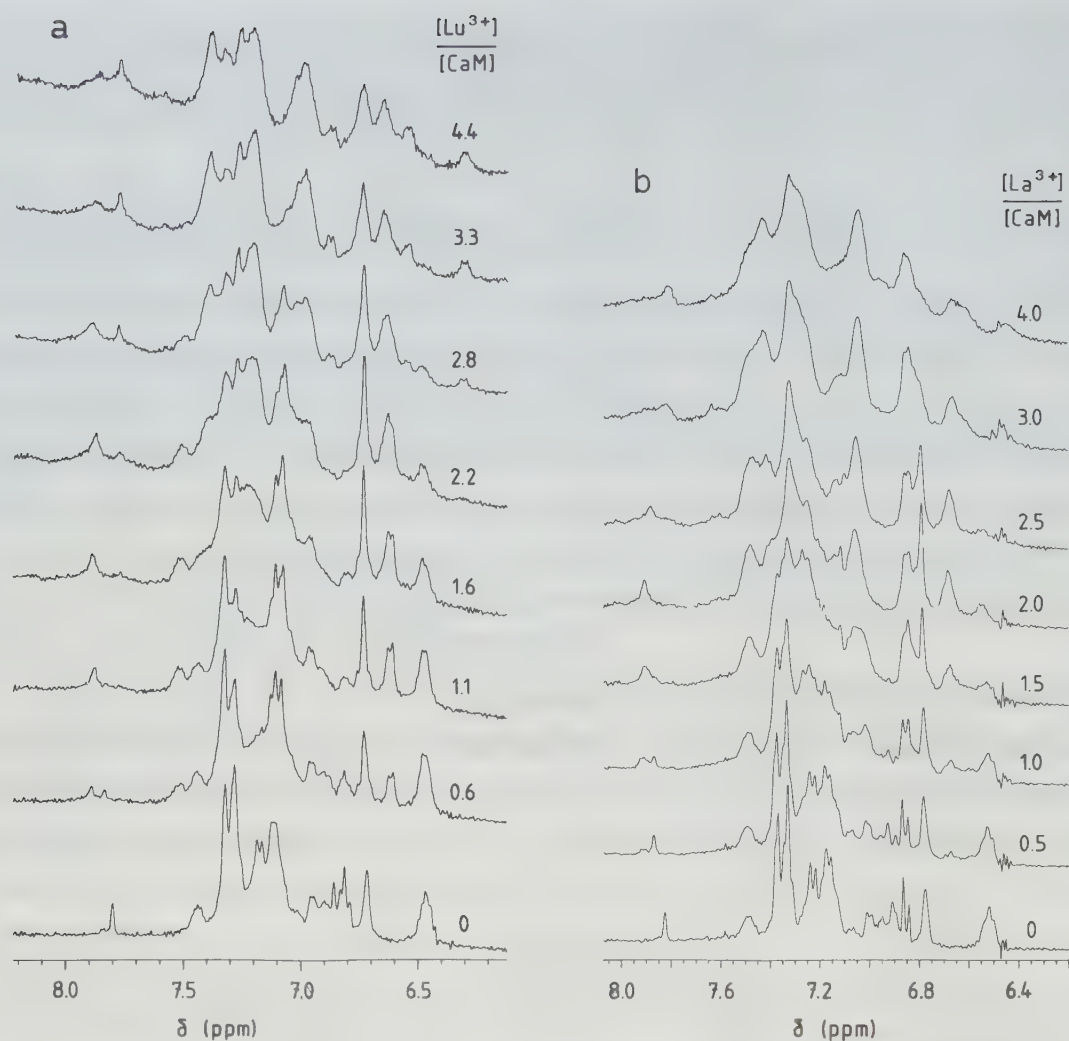


Figure 3. Aromatic region of the 360 MHz ^1H NMR spectrum of calmodulin (2.0 mM, pH 7.5) (a) titrated with Lu^{3+} and (b) titrated with La^{3+} . Note that the series of spectra are in slow exchange. 500 scans.

encountered during the whole Ln^{3+} titration. By contrast, two phases (slow exchange during the first half and fast exchange during the second half) are observed in the Ca^{2+} titration. Moreover, resonances characteristic of group I above belong to stage II in the Ca^{2+} titration (changes occur during the second half of the Ca^{2+} titration). Similarly, resonances typical of group II belong to stage I in the Ca^{2+} titration. In addition, the three-dimensional structure of Lu^{3+} - or La^{3+} -saturated calmodulin must differ from the structure of Ca^{2+} -saturated calmodulin as the ^1H NMR spectra of Lu^{3+} - or La^{3+} -saturated CaM exhibit large differences compared to ^1H NMR spectrum of Ca^{2+} -saturated CaM. Thus, it is possible to conclude that Lu^{3+} and La^{3+} ions are not bound to CaM in the same order as Ca^{2+} ions and do not induce the same conformational change of CaM as Ca^{2+} ions. Recent stopped-flow studies have also provided evidence that Ca^{2+} and Tb^{3+} have different binding preferences toward the four Ca^{2+} binding sites of CaM [Wang et al., 1984; Linse et al., 1986].

3.3. Kinetics of Ca^{2+} Binding to Calmodulin

The kinetics of Ca^{2+} binding to calmodulin and its tryptic fragments have been studied by stopped-flow fluorescence (paper III) and ^{43}Ca NMR (paper IV). Interestingly, it appears that Ca^{2+} dissociation from CaM occurs in two distinct steps. The two rate processes, each involving two calcium ions, are resolved for CaM at both low and high ionic strength. The effect of 0.1 M KCl is to accelerate the slow process from $9.1 \pm 1.5 \text{ s}^{-1}$ to $24 \pm 6.0 \text{ s}^{-1}$ and to reduce the rate of the fast process from 650 s^{-1} to $240 \pm 50 \text{ s}^{-1}$ at 25°C , paper III. Similar values were obtained by ^{43}Ca NMR measurements, paper IV. Activation parameters for the two processes were determined. The exchange rates have negative entropies of activation.

The kinetics of Ca^{2+} binding to CaM are also strongly affected by added hydrophobic drugs, cf. section 4.1. The Ca^{2+} dissociation rates from CaM are markedly reduced in the presence of the hydrophobic drug trifluoperazine, paper III.

Studies of the tryptic fragments TR_1C and TR_2C clearly identified Ca^{2+} binding sites I and II as the rapidly exchanging (low-affinity) sites and sites III and IV as the slowly exchanging (high-affinity) sites. The kinetic properties of the two proteolytic fragments are closely similar to the fast and slow dissociating sites of native CaM.

4. Drug Binding to Calmodulin

Binding of calcium ions to calmodulin causes the exposure of hydrophobic domains on the surface of the protein that appear to be essential for the interaction of CaM with its target proteins [for a recent review see Forsén et al., 1986]. Various drugs of hydrophobic nature, like the antipsychotic drug trifluoperazine (TFP) that strongly inhibit the biological activity of CaM, are considered to compete with target proteins for the same binding sites on CaM's surface. The mode of binding of small drug molecules and the location of their binding sites on CaM are therefore of considerable interest. We have examined binding of some common drugs to CaM under various conditions and the binding of TFP to CaM has been studied in some detail. Since some of these studies have not been reported earlier they are described at some length below.

4.1. General Effects of Drug Binding to Calmodulin

Binding of drugs to calmodulin affects all four cation binding sites as inferred from studies using ^{113}Cd NMR (papers II, V and VI) and stopped-flow fluorescence (paper III). The structural rearrangements that take place upon binding of drugs are thus transmitted to the domains in the protein where Ca^{2+} is bound. The most pronounced effects that occur in ^{113}Cd NMR spectra of CaM upon binding of drugs are: (1) an upfield displacement of the chemical shift for the two

signals in slow exchange; (2) a drastic reduction of the exchange rate for the $^{113}\text{Cd}^{2+}$ ions undergoing fast to intermediate exchange, so that two additional narrow signals appear in the spectrum.

The drugs are assumed to bind to the same interaction sites (except felodipine). In fact, TFP replaces D600 from at least one binding site, paper V. Similar chemical shifts are obtained for the different spectra of drug-saturated Cd_4CaM , paper VI. Since ^{113}Cd NMR chemical shifts are known to be extremely sensitive to small changes in protein structure [Cavé et al., 1982] the drug molecules studied, in spite of their seemingly structural discrepancy (see Table 2), most likely induce the same overall changes in the conformation of the protein.

The fact that two additional ^{113}Cd NMR signals appear upon drug binding to Cd_4CaM indicates that the cation affinity for the two weak metal ion binding sites is increased upon drug binding. Indeed, a reduced Ca^{2+} dissociation rate is measured by the stopped-flow technique when TFP is added to CaM, paper III. Further evidence in support of increased affinity of CaM for Ca^{2+} in the presence of TFP has been provided by ^{43}Ca NMR experiments [Vogel et al., 1984]. In order to determine the relative cation affinity for the four cation binding sites in the presence of drug both a Cd^{2+} titration and a Ca^{2+} titration were performed. The results are presented below.

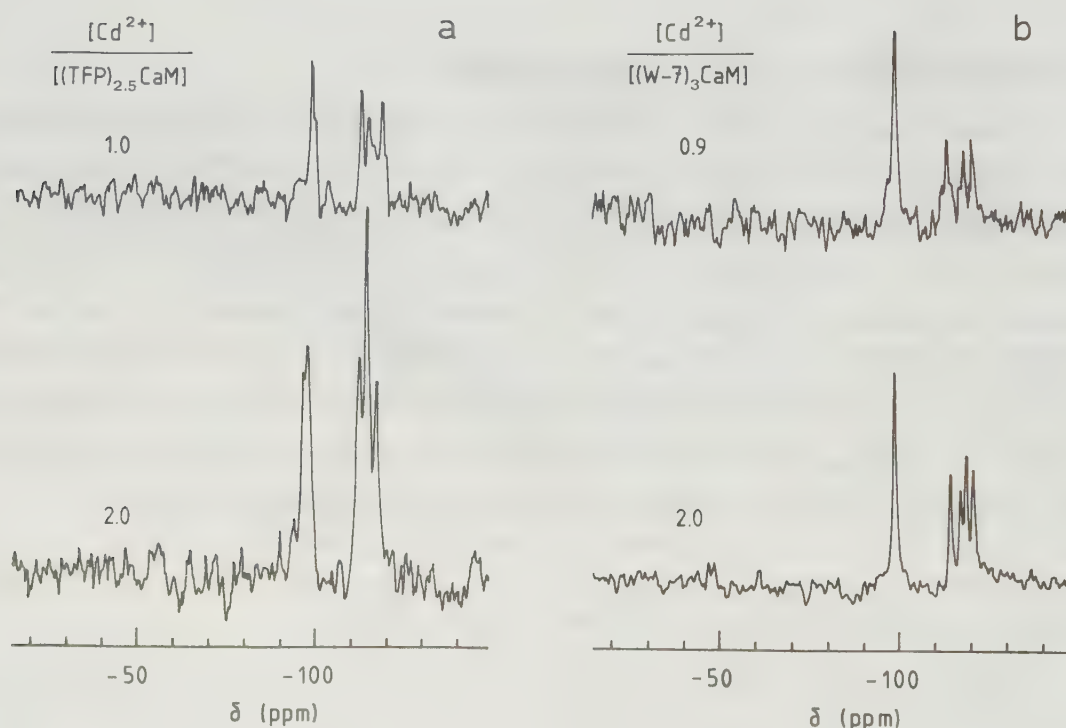


Figure 4. Changes in the ^{113}Cd NMR spectrum of a solution containing drug-saturated CaM upon Cd^{2+} addition. (a) 1.3 mM CaM, 3.2 mM TFP, pH 7.0 (b) 1.4 mM CaM, 4.2 mM W-7, pH 7.0.

When Cd^{2+} ions are added to apo calmodulin in the absence of drug only two resonances are observed in the ^{113}Cd NMR spectrum up to a ratio of $\text{Cd}^{2+}:\text{CaM}$ of 4:1, cf. Figure 1 paper VI. In the presence of a drug, up to five resonances are resolved at low Cd^{2+} to CaM ratios, Figure 4. The values of the chemical shift for the resonances indicate that the drug molecules are bound to CaM. Three kinds of species may exist already at low Cd^{2+} to CaM ratios: CaM-drug complex, Cd_2CaM -drug complex and Cd_4CaM -drug complex. Thus, TFP and W-7 strengthen the binding of Cd^{2+} to the low-affinity cation sites to a value comparable to that for the high-affinity cation sites.

When Ca^{2+} ions are added to TFP-saturated calmodulin a slow exchange behaviour is encountered for the protein resonances in the ^1H NMR spectrum during the whole titration. The signals from TFP partly overlap those of CaM which renders it difficult to follow the normally well resolved resonances from aromatic residues of CaM. However, the relative intensity of the resonances from Tml-115 (in the Ca^{2+} -free state at 3.13 ppm and in the Ca^{2+} -saturated state at 3.11 ppm) can be measured with high accuracy. A decrease (and increase) is observed during the whole Ca^{2+} titration, Figure 5. Other resolved resonances go through a similar change. Thus, only one stage with slow exchange is observed during the Ca^{2+} titration in the presence of TFP, contrary to the two stages (one with slow and one with fast exchange) observed in the absence of drug. Therefore CaM exists mainly as CaM-TFP complex and Ca_4CaM -TFP complex in the presence of

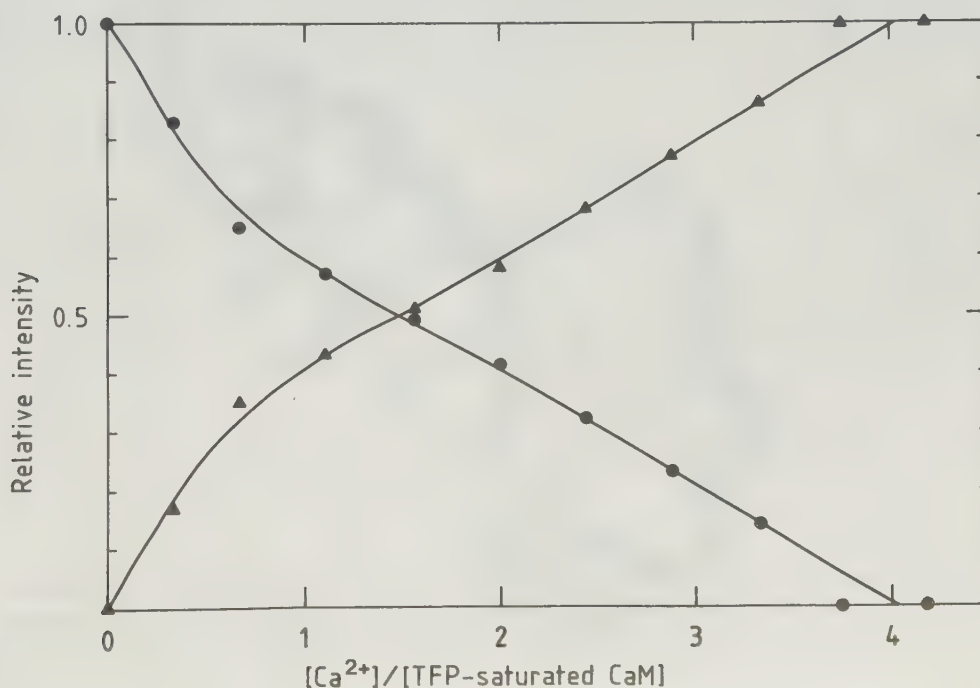


Figure 5. 360 MHz ^1H NMR relative intensities measured for the Tml-115 signals, (●) in the Ca^{2+} -free state and (▲) in the Ca^{2+} -saturated state, as Ca^{2+} is added. Conditions used: 1.7 mM CaM, 5.0 mM TFP, pH 7.0.

drug. The Ca^{2+} affinity of the low-affinity sites seems to change in the presence of drug so that the four sites have an almost equal Ca^{2+} affinity. The deviation from linearity, cf. Figure 5, indicates that a small amount of $\text{Ca}_2\text{CaM-TFP}$ complex exists in the solution.

4.2. Binding of Trifluoperazine to Calmodulin

The drug trifluoperazine, Figure 6, has provided an extremely useful tool for the study of the Ca^{2+} -exposed hydrophobic surfaces of CaM. Large changes in both ^{113}Cd NMR spectra and Ca^{2+} dissociation rates as measured by stopped-flow fluorescence are observed upon addition of TFP to CaM, papers II, III, V and VI. The two strong binding sites for the TFP molecule of CaM are largely intact in the fragments, paper II. One binding site is located on the amino-terminal half and the other on the carboxy-terminal half. The binding constants for the two strongly bound TFP molecules differ, the strongest TFP binding site being located on the carboxy-terminal half.

Further studies have been performed using ^{19}F NMR. Addition of Ca^{2+} -saturated calmodulin to a sample of trifluoperazine displaces and broadens the $-\text{CF}_3$ resonance from TFP in the ^{19}F NMR spectrum, Figure 7. Only one resonance is observed at all studied ratios of TFP to CaM. The chemical exchange of TFP molecules between free and bound to CaM is thus fast on the ^{19}F NMR time scale.

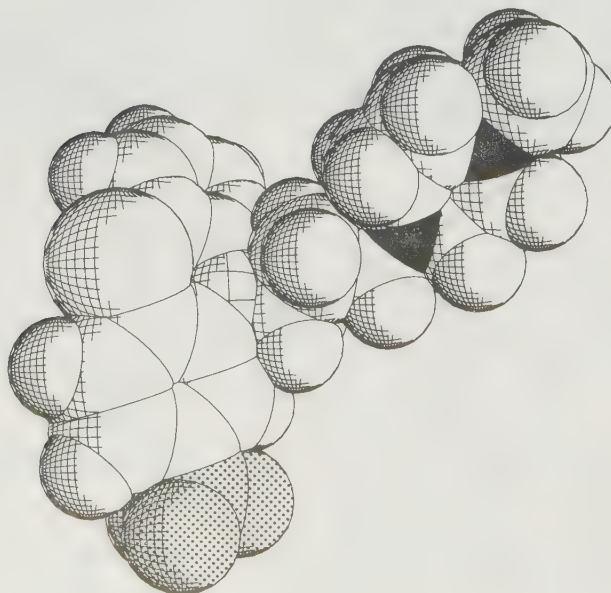


Figure 6. Space-filled model of trifluoperazine. Atomic coordinates were obtained from McDowell [1980]. The two nitrogens in the piperazine ring are marked black and the $-\text{CF}_3$ group is dotted.

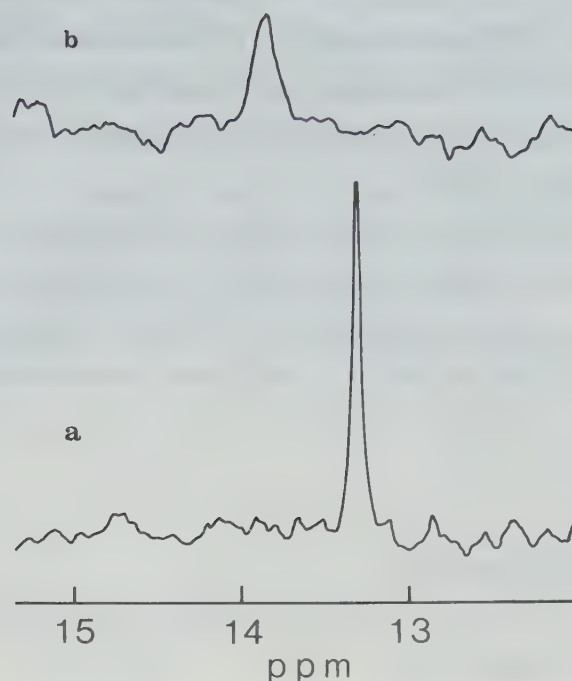


Figure 7. 94.1 MHz ^{19}F NMR spectra of TFP (0.5 mM, pH 6.8) before (a) and after (b) addition of 2 equivalents of Ca^{2+} -saturated CaM. The chemical shift is measured relative to trifluoroacetic acid. 300 scans.

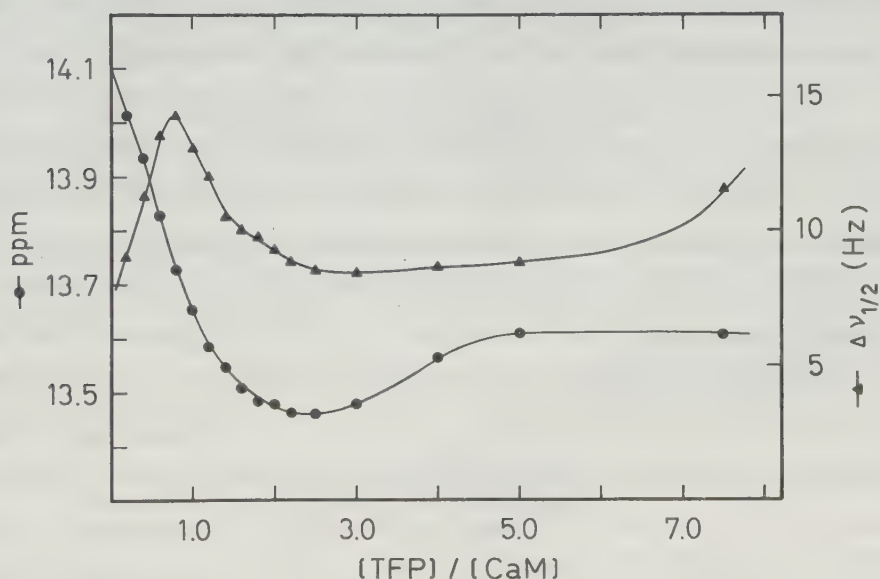


Figure 8. Chemical shift (●) and line width at half-height (▲) of the TFP resonance in the ^{19}F NMR spectrum during a TFP titration to 1.1 mM Ca^{2+} -saturated CaM, pH 7.0. The number of scans varied from 1.68×10^5 at 0.2 equivalents of TFP to 1000 at 7.5 equivalents of TFP.

The changes in chemical shift and line width of the $-\text{CF}_3$ resonance upon titration of Ca^{2+} -saturated CaM with TFP are shown in Figure 8. An upfield displacement of the chemical shift

is observed between 0 and 2 equivalents of TFP per CaM. The chemical shift goes through a minimum of 13.4 ppm at a TFP:CaM ratio of 2.5:1. A final chemical shift (13.6 ppm) seems to be reached at 5 equivalents of TFP. A maximum line-broadening of 6 Hz to a line width of 14 Hz is observed at a TFP:CaM ratio of 0.8:1. A precipitate was formed and the line width concomitantly increased above 5 equivalents of TFP.

In the ^{19}F NMR chemical shift range the change observed of a maximal 0.65 ppm is small, but still significant. From the chemical shift variation it is possible to conclude that the binding constants for the two strong TFP binding sites differ. The downfield change in chemical shift in the later part of the titration indicates binding to the many weak calcium-independent TFP sites.

5. Concluding Remarks

Results presented in this thesis show how well suited calmodulin is to function as a calcium-binding regulator protein.

The four Ca^{2+} binding sites can be grouped into two with higher and two with lower affinity, the difference being most pronounced at low ionic strength. Positive cooperative binding of the first two Ca^{2+} ions to domains III and IV induces an exposure of a hydrophobic carboxy-terminal interaction site. This might be sufficient to establish binding between CaM and a target enzyme but not necessarily activation. As the last two Ca^{2+} ions bind to domains I and II a second hydrophobic interaction site occurs, now on the amino-terminal half. This might confer the regulatory action of the activity. Binding of drugs to the hydrophobic interaction sites seems to enhance the Ca^{2+} affinity of all four domains. The most pronounced effect occurs on Ca^{2+} binding domains I and II so that the difference in affinity between the two groups of Ca^{2+} binding sites is reduced. Various studies have shown that CaM's affinity for Ca^{2+} is markedly increased in ternary complexes of Ca^{2+} -CaM-protein [Rasmussen, 1983; Keller et al., 1982; Maulet and Cox, 1983; Olwin and Storm, 1985]. It is thus reasonable to presume that an enhancement of the Ca^{2+} affinity of domains I and II occurs in such ternary complexes in a similar way as is observed when drugs bind. The positive cooperativity between Ca^{2+} binding domains III and IV and the reduced difference in Ca^{2+} affinity between the two groups of Ca^{2+} binding sites when CaM interact with drugs and/or target enzymes results in a steeper Ca^{2+} -binding curve. This allows CaM to modulate target proteins in a much narrower range of Ca^{2+} concentration than otherwise possible.

From observed Ca^{2+} -binding constants [Klee and Vanaman, 1982] and Ca^{2+} exchange rates determined in our laboratory it is possible to conclude that the Ca^{2+} on-rates to calmodulin are close to diffusion limited. CaM is thus able to rapidly respond to fluctuations in the intracellular Ca^{2+} level. This is in contrast to extracellular calcium-binding proteins that generally have much slower

on- and off-rates [Vogel and Forsén, 1986].

Although the two tryptic fragments bear a remarkable resemblance to intact CaM, it would be too simple to suggest that CaM is nothing more than the sum of these two parts. Binding of TFP to the TR₁C fragment appears to be weaker than binding of this hydrophobic drug to the amino-terminal half of CaM, papers II and III. Moreover the fragments can not mimic the modulating capabilities of CaM [Walsh et al., 1977; Kuznicki et al., 1981; Schreiber et al., 1981; Newton et al., 1984]. Thus the interplay between CaM and other molecules seems to be dependent on the presence of the unbroken native protein, allowing for interactions between the two globular halves of CaM.

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The author

Lund, March 26, 1986

Paper I

Cadmium-113 Nuclear Magnetic Resonance Studies of Proteolytic Fragments of Calmodulin: Assignment of Strong and Weak Cation Binding Sites[†]

Anita Andersson, Sture Forsén, Eva Thulin, and Hans J. Vogel*

ABSTRACT: Proteolytic fragments of bovine testis calmodulin were obtained by limited proteolysis with trypsin or thrombin. Cadmium-113 NMR studies showed that the tryptic fragment encompassing Ca^{2+} binding domains III and IV (TR_2C) gave rise to a spectrum identical with that of the native protein. Two thrombinic fragments containing either domains I, II, and III [TM_1 -(1-106)] or the single domain IV [TM_2 -(107-148)] both gave rise to one broad resonance only. These data in-

dicate that domains III and IV comprise the two high-affinity Ca^{2+} binding sites in intact calmodulin and that disturbance of the structural relationship between domain III and domain IV markedly reduces the affinity of these two sites for Ca^{2+} ions. These observations are discussed with respect to other published accounts concerning the sequence in which the four calcium domains in calmodulin are filled.

The effects of the intracellular messenger Ca^{2+} are often mediated through the heat- and acid-stable protein calmodulin (M_r 16 700). CAM¹ usually interacts with its receptor proteins only after binding of calcium ions [for reviews see Cheung (1980), Klee (1980), and Klee et al. (1980)]. Binding of up to four calcium ions causes large conformational changes in the protein. Amino acid sequencing studies have revealed the presence of four EF handlike calcium binding domains (Vanaman, 1980). Although many studies have addressed the question of the affinity of these four separate sites for Ca^{2+} ions, unfortunately no unique answer has emerged yet [see, for example, Klee (1980)]. Moreover, no clarity exists at present as to how many Ca^{2+} ions are bound in CAM-receptor protein complexes and, in addition, there is still considerable debate as to the order in which the sites are filled in purified apo-CAM (see Discussion).

Here we wish to describe our ^{113}Cd NMR studies comparing the binding of Cd^{2+} to calmodulin and some of its proteolytic fragments that are obtained by limited proteolysis. Studies in our laboratory with at least six different calcium binding proteins have shown that the Cd^{2+} ion is a convenient and reliable probe in the study of calcium binding sites [for review see Vogel et al. (1983)]. Our earlier studies with calmodulin (Forsén et al., 1980; Andersson et al., 1982) have shown that

calmodulin contains two "high-affinity" sites that give rise to two resolved resonances in a ^{113}Cd NMR spectrum, whereas the two "low-affinity" sites are not observed due to exchange between free² and protein-bound Cd^{2+} ions.³ Only after addition of drugs that are known to bind to the hydrophobic surface of calcium or cadmium-saturated CAM is this exchange rate reduced and two additional resonances for the other two sites appear in the spectra (Forsén et al., 1980; Sudmeier et al., 1980; A. Andersson, unpublished results).

Experimental Procedures

Materials. Calmodulin was prepared from bovine testis essentially as described by Jamieson & Vanaman (1979). Apo-CAM was prepared by passage of CAM over a Chelex-

¹ Abbreviations: CAM, calmodulin; NaDodSO₄, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance; UV, ultraviolet; CD, circular dichroism; TPCK, 1-(tosyl-amido)-2-phenylethyl chloromethyl ketone; Tris, tris(hydroxymethyl)-aminomethane; DTT, dithiothreitol; DEAE, diethylaminoethyl.

² The term "free Cd" is only a relative nomenclature. Cd^{2+} ions will interact weakly with (all) NH sites on the surface of any protein, resulting in a very broad resonance for these "free" ions that is usually less sharp than those for the tightly protein-bound Cd^{2+} ions. Cd^{2+} ions also interact with ions like Tris or Cl^- , which also cause changes in the Cd line width and chemical shift; hence, results are more easily interpretable when no buffer is used.

³ Another possibility is a fast exchange between two conformations with different chemical shifts for these two metal ions.

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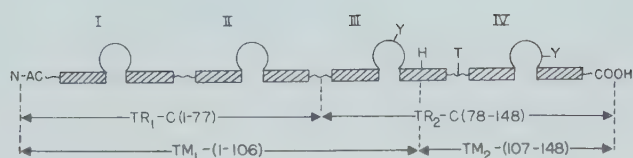


FIGURE 1: Schematic representation of the proposed structure of calmodulin outlining the four domains, each comprising a calcium binding loop and two flanking helix regions (hatched areas). Also indicated are the positions of the two tyrosine residues at positions 99 and 138 (Y) and the positions of the single histidine-106 (H) and trimethyllysine-115 (T) residues. Proteolytic fragmentation as induced by tryptic (TR) or thrombin (TM) cleavage is also indicated.

100 column; the residual amount of metal ion was determined by ^1H NMR experiments and was always below 0.1. TPCK-treated trypsin, thrombin (grade I), and soybean trypsin inhibitor (type I-S) were obtained from Sigma Chemical Co. ^{113}CdO (96.3% enriched in isotope ^{113}Cd) was obtained from Oak Ridge National Laboratory. All other chemicals used were of analytical grade and were obtained from local suppliers.

Methods. Thrombin fragments of calmodulin were prepared following the method of Wall et al. (1981). Calmodulin (10 mg) was incubated for 2 h at 37 °C and pH 8.7 (50 mM Tris, 5 mM EDTA, and 1 mM DTT) with 100 units of thrombin. The peptides thus obtained were subsequently purified on a Sephadex G-50 column. Proteolytic fragmentation with trypsin followed essentially the procedures described by Drabikowski et al. (1977) and Walsh et al. (1978). Typically 1 mg of trypsin was added to 100 mg of CAM dissolved in 100 mL of freshly prepared 100 mM NH_4HCO_3 –1 mM CaCl_2 , pH 7.8. The reaction was stopped after 35 min at room temperature by the addition of 2 mg of soybean trypsin inhibitor. Fragments were purified on a DEAE-Sephadex A-25 column equilibrated in 50 mM KH_2PO_4 , pH 7.2, which was eluted with a linear NaCl gradient up to a concentration of 0.5 M. Under these conditions CAM had completely disappeared, and peptide TR_2C (see below) was the last to elute from the column. Peptides were checked for purity by NaDodSO₄ gel electrophoresis and agarose gel electrophoresis in the presence and absence of EDTA or Ca^{2+} . Moreover, their amino acid composition was checked by ^1H NMR (361-MHz Nicolet WB) using the resonances for the two tyrosines and the single trimethyllysine and histidine residues as indicators (Seamon, 1980; Krebs & Carafoli, 1982). Figure 1 indicates the position of these moieties in intact calmodulin, and it can readily be seen that all four indicated fragments can be identified in this fashion.

^{113}Cd NMR spectra were obtained at room temperature on a home-built spectrometer equipped with an Oxford instrument 6-T magnet operating at a frequency of 56.55 MHz. A home-built probe with a horizontal sample orientation was used for these studies. Proteolytic fragments or whole CAM was dissolved in doubly distilled water treated with Chelex-100. The pH of all samples was kept between 7.0 and 7.5. Typical acquisition parameters have been described elsewhere (Forsén et al., 1979, 1980).

Results

Proteolytic Fragmentation. Limited tryptic digestion of CAM in the presence of saturating amounts of Ca^{2+} will result in cleavage only at lysine-77, thus giving rise to two peptides of equal size (see Figure 1) (Walsh et al., 1977; Drabikowski et al., 1977, 1982). Under our experimental conditions (see Methods) we found that peptide TR_2C accumulated, whereas peptide TR_1C was partially further degraded. Similar results

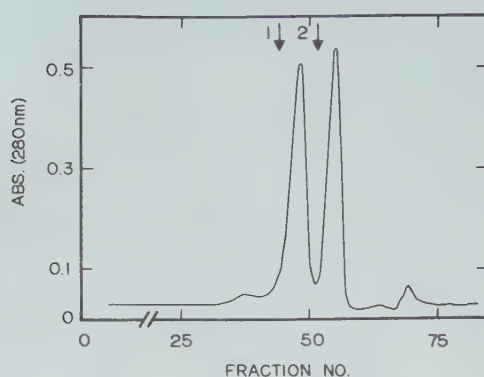


FIGURE 2: Column profile on Sephadex G-50 obtained after thrombin cleavage of CAM. Indicated by the arrows are the elution positions of CAM (1) and fragment TR_2C (2). See text for further explanation.

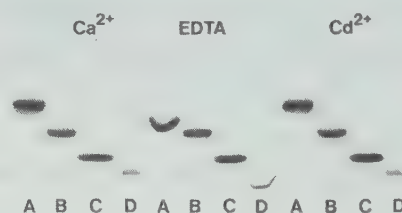


FIGURE 3: NaDodSO₄ gel electrophoresis of CAM and its proteolytic fragments (10–20% gradient gels, run at pH 8.7). Identification of the bands is as follows: (A) CAM; (B) TM_1 ; (C) TM_2 ; (D) TR_2C .

have been described by others (Klee, 1980).

Incubation of CAM with thrombin results in a highly selective cleavage at arginine-106, giving rise to two fragments of different size (see Figure 1) that can be readily fractionated on a Sephadex G-50 column (see Figure 2) (Wall et al., 1981). The small shoulder preceding the first peak probably comprises residual thrombin, whereas the first peak contains fragment TM_1 and the second fragment, TM_2 . Also indicated in Figure 2 are the elution positions of CAM and fragment TR_2C on the same column, indicating that the relative size of the fragments is as expected from Figure 1.

Electrophoretic Studies. In order to assess the affinities of these fragments for Ca^{2+} and Cd^{2+} , we studied their behavior in the presence and absence of Ca^{2+} , Cd^{2+} , and/or EDTA on NaDodSO₄ gel electrophoresis. Similar experiments have been described in order to assess the binding of lanthanides to CAM (Wallace et al., 1982). It has been known for some time that abnormally high values for the molecular weight can be obtained for various highly acidic calcium binding proteins [see Klee (1980) and references cited therein] and that the mobility can be different in the presence of Ca^{2+} or chelating agents. As can be seen in Figure 3, such behavior is found most notably for peptide TM_2 , which runs on the NaDodSO₄ gel as if it has a molecular weight between TM_1 and TR_2C . From the results in Figure 3 it is clear that for CAM and TR_2C different mobilities are observed when EDTA or cation is present. It is important to note that no difference between Ca^{2+} and Cd^{2+} is observed, showing that Cd^{2+} is a good substitute for Ca^{2+} . Note further that the mobility of peptides TM_1 and TM_2 is not affected by the addition of cation or chelator.

^{113}Cd NMR Studies of Fragment TR_2C . Parts a and b of Figure 4 compare the ^{113}Cd NMR spectra obtained after saturation of CAM and TR_2C with Cd^{2+} . In both cases two sharp resonances are observed at chemical shift positions -87.5

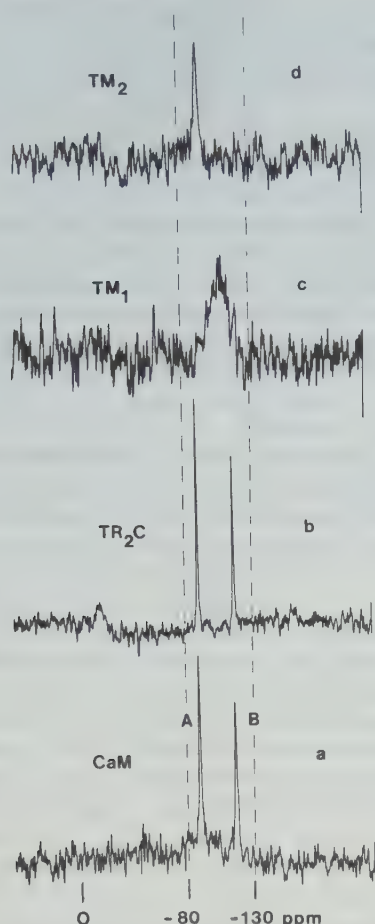


FIGURE 4: ^{113}Cd NMR spectra of calmodulin (a) and its fragments (b-d). Conditions used were as follows: (a) 1.2 mM CAM, 4.8 mM Cd^{2+} , 2.5×10^4 acquisitions; (b) 0.3 mM TR_2C , 0.6 mM Cd^{2+} , 3×10^5 acquisitions; (c) 0.92 mM TM_1 , 2.7 mM Cd^{2+} , 5×10^4 acquisitions; (d) 1.30 mM TM_2 , 2.5 mM Cd^{2+} , 5×10^4 acquisitions.

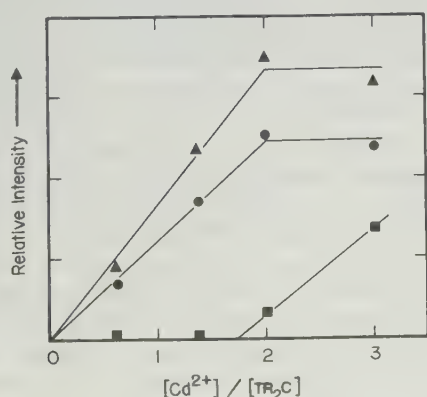


FIGURE 5: Increase in signals A (▲), B (●), and free Cd^{2+} (■) upon titration of apo- TR_2C (0.3 mM) with Cd^{2+} (other conditions as in Figure 4b).

and -114.2 ppm.⁴ Thus we conclude that resonances A and B in the intact protein represent Cd^{2+} bound to domains III and IV. Figure 5 shows the peak intensities observed for both signals upon $^{113}\text{Cd}^{2+}$ titration of apo- TR_2C . Clearly, as with CAM (Forsén et al., 1980), both signals increase in parallel rather than sequentially, indicating either that domains III and IV have identical binding constants or that binding occurs in a positive cooperative fashion (Klee, 1977; Crouch & Klee,

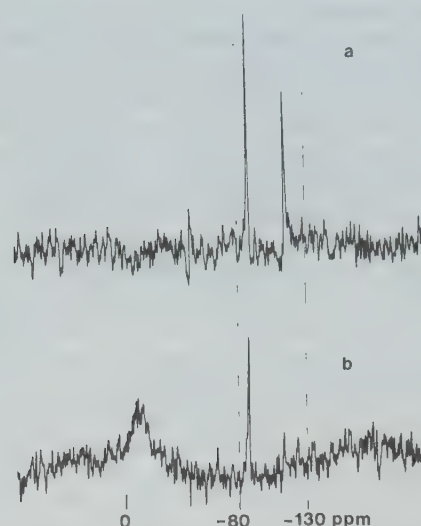


FIGURE 6: ^{113}Cd NMR spectra of Cd^{2+} -saturated TR_2C (0.3 mM) before (a) and after (b) addition of 1.3 equiv of Ca^{2+} (1.3×10^5 acquisitions).

1980). Since we have observed the same parallel increase for skeletal and cardiac troponin C, we feel that positive cooperativity is the more likely explanation.

^{113}Cd NMR Studies with Thrombic Fragments. Parts c and d of Figure 4 show the spectra obtained with fragments TM_1 and TM_2 after the addition of 3 and 2 equiv of Cd^{2+} ions, respectively. The broad signal for TM_1 appears at -109 ppm and probably represents binding to domain III; the signal for TM_2 (domain IV) appears at -95 ppm. Unfortunately, the proteolytic cleavage of the protein between domain III and domain IV has caused changes in the calcium binding loops III and IV such that the chemical shift positions for the thrombic fragments no longer correspond to those observed for CAM. However, since Cd^{2+} bound to TM_1 resonates closest to B and that of TM_2 resonates closest to A, it is tempting to speculate that resonances A and B represent Cd^{2+} bound to domains IV and III, respectively, in CAM. Spectra obtained at lower and higher ratios of Cd^{2+} with respect to thrombic peptide gave essentially the same chemical shifts. The resonances for TM_1 are somewhat sharper when 2 or 4 equiv are present, whereas the resonance for TM_2 becomes broadened somewhat when more Cd^{2+} is added (data not shown).

Metal Ion Competition. Figure 6 shows the results obtained after the addition of 1.3 equiv of CaCl_2 to a sample of Cd^{2+} -saturated TR_2C . Addition of only 1 equiv abolishes resonance B whereas after addition of 2.7 equiv about 40% (and after addition of 4.0 equiv about 25%) of resonance A is still present (data not shown). Identical results have been reported for intact bovine testis CAM (Forsén et al., 1980).

Discussion

Recent studies with proteolytic or synthetic fragments of calcium binding proteins have provided some new insights in the functioning of calcium binding proteins. In general, it has been observed that fragments containing only one binding domain (like TM_2) have lower affinity for Ca^{2+} than fragments containing a pair of related domains (like TR_2C). The latter behave often very similar to the intact protein [for review and discussion see Seamon & Kretsinger (1983) and Vogel et al. (1983)]. For example, in the case of skeletal troponin C, a protein which is very similar with CAM, studies with fragments demonstrated unequivocally that domains III and IV contain the two high-affinity Ca^{2+} binding sites [Leavis et al.,

⁴ The difference in intensity between signal A and signal B arises from a slight difference in T_1 for these two sites.

1978; for discussion see McCubbin & Kay (1980)]. Spectroscopic studies provided evidence that the structural characteristics are maintained in some of these fragments and that Ca^{2+} binding often gives rise to similar changes in the spectroscopic parameters as for the intact protein (Leavis et al., 1978; Evans et al., 1980). Although CAM and skeletal troponin C are homologous (Vanaman, 1980), the situation is less clear as to the location of the two high-affinity sites in CAM. Estimates of the dissociation constants have indicated that all four sites have dissociation constants within one order of magnitude ($K_D \approx 10^{-5}$ – 10^{-6} M) and that the four sites can be divided into two subclasses, two with lower and two with higher affinity (Klee, 1980). Earlier ^{113}Cd and ^{43}Ca NMR studies have also provided evidence for the existence of two high-affinity and two low-affinity sites. The former are characterized by a much slower metal ion off-rate than the latter⁵ (Fors  n et al., 1980; Andersson et al., 1982). The experiments reported here show unequivocally that the two sites with slower metal off-rates are domains III and IV, since the ^{113}Cd NMR spectra obtained with the tryptic fragment TR_2C are identical with those obtained with CAM (compare parts a and b of Figure 4). Since ^{113}Cd NMR chemical shifts are known to be extremely sensitive to small changes in protein structure, as exemplified by studies on a series of homologous parvalbumins (Cav   et al., 1982), the structure of the binding sites in CAM and TR_2C must be identical. Note that *no* two sharp resonances are observed for peptide TM_1 (containing domains I, II, and III) (see Figure 4c), suggesting that domains I and II contain the protein bound Cd^{2+} ions that are not observable (due to exchange broadening) for CAM (Figure 4a; Fors  n et al., 1980). Rather, we observed only one broad resonance, probably representing the Cd^{2+} bound to domain III. Also, for TM_2 (domain IV) we only observed one broadened resonance, although the broadening here is not as marked as observed for TM_1 . This broadening effect for these two resonances is of interest. In these particular experiments the line width can be viewed as approximately inversely related to the affinity:⁵ thus the order of affinity $\text{CAM} > \text{TM}_2 > \text{TM}_1$. The notion that TM_2 has a higher affinity for metal ions than TM_1 is of interest since studies with synthetic peptides have shown that the presence of an α -helix on either side of the calcium binding loop increases the affinity for Ca^{2+} ions (Reid et al., 1981). TM_1 has only three amino acids on its carboxy-terminal end of the calcium binding loop, which are capable of forming an α -helix, whereas the calcium binding loop of TM_2 is surrounded by its native α -helices, and the latter is thus expected to have a higher affinity for the metal ion. In addition, it should be noted that both TM_1 and TM_2 have a lower affinity for Cd^{2+} than peptide TR_2C . From the data presented in Figure 3 it is also clear that both Ca^{2+} and Cd^{2+} have a lower affinity for the thrombic fragments, since their electrophoretic mobility is not altered upon addition of metal ion or chelator. This is consistent with the idea that a pair of related calcium binding domains has a higher affinity than one isolated domain (Seamon & Kretsinger, 1983; Vogel et al., 1983). In this respect it is of interest that the only two crystal structures reported to date for calcium binding proteins (Kretsinger & Nockolds, 1973; Szebenyi et al., 1981) show not only that the structure of the calcium binding loops and the surrounding helices are conserved but also the approximate 2-fold symmetry within a pair of domains. This structural

relationship is also likely to be important for the cooperativity between the two high-affinity sites (Klee, 1977; Crouch & Klee, 1980; Fors  n et al., 1980). Upon titration of apo-CAM (Fors  n et al., 1980) or apo- TR_2C (Figure 5) with Cd^{2+} , signals A and B increase in parallel in both cases, suggesting positively cooperative interactions between domain III and domain IV that are identical in the peptide and in intact CAM.

It could be argued that affinity for Ca^{2+} and Cd^{2+} ions is not similar. Although both ions have the same charge and a very similar size (0.097 and 0.099 nm, respectively), some calcium binding proteins have a higher (Drakenberg et al., 1978) and some a lower (Murakami et al., 1982) affinity for Cd^{2+} than for Ca^{2+} . Several lines of evidence suggest however that Cd^{2+} is a good probe in the studies with proteolytic fragments outlined above. First, our studies with NaDodSO_4 gel electrophoresis show that Ca^{2+} and Cd^{2+} induce the same change in mobility. Second, the metal ion competition between Ca^{2+} and Cd^{2+} observed with TR_2C (Figure 6) is identical with that observed for the intact protein (Fors  n et al., 1980). Third, two sites with high and low affinity are observed for CAM both in "perturbing" ^{113}Cd NMR and in "nonperturbing" ^{43}Ca NMR studies, indicating the high similarity between the outcome of experiments with the two different probes. Studies with ^{43}Ca NMR of proteolytic fragments are presently in progress in our laboratory. Finally, ^1H NMR titration studies with both Ca^{2+} and Cd^{2+} show identical changes in resolved resonances for intact calmodulin as well as for the proteolytic fragments (our unpublished results).

Our results discussed above indicate that III and IV are the first domains to be filled in intact apoprotein. Various other studies have been reported addressing this same problem. UV and near-UV-CD experiments, fluorescence experiments, and ^1H NMR measurements of CAM indicate that tyrosines-99 and -138 that are located in calcium binding loops III and IV (see Figure 1) are most effected by binding of the first two calcium ions and are thus consistent with the notion that domains III and IV comprise the two high-affinity sites (Klee, 1977; Crouch & Klee, 1980; McCubbin et al., 1979; Dedman et al., 1977; Seamon, 1980; Krebs & Carafoli, 1982). In addition, UV, CD, and fluorescence studies (Drabikowski et al., 1977, 1982; Walsh et al., 1977) as well as ^1H NMR studies (our unpublished results) with the proteolytic fragment TR_2C show that very similar, if not identical, perturbations for the tyrosines are observed upon binding of Ca^{2+} ions, thus providing further evidence for this notion. However, in strong contrast to the above, flow dialysis experiments (Haiech et al., 1981), ^{23}Na NMR measurements (Delville et al., 1980), and most particularly terbium luminescence transfer experiments (Kilhoffer et al., 1980a,b; Wang et al., 1982; Wallace et al., 1982) provided evidence for another sequence in which the four sites are filled. It should be recalled that in these studies monovalent ions (Haiech et al., 1981; Delville et al., 1980) or trivalent metal ions were used as probes, and thus it has been alleged that these may behave differently from Ca^{2+} (Seamon & Kretsinger, 1983). Indeed, monovalent cations are incapable of inducing Ca^{2+} -like conformational changes in the protein (Klee, 1980), and thus the affinity for these does not necessarily have to parallel that of Ca^{2+} . Moreover, the suggestion that K^+ will bind strongest to the site with the most carboxylate groups is not necessarily correct (Haiech et al., 1981). Since Tb^{3+} is generally considered to be a reasonable replacement for Ca^{2+} (Martin & Richardson, 1979), it is more difficult to rationalize why the results obtained with Tb^{3+} luminescence transfer are out of line with the evidence dis-

⁵ The metal-exchange rates are only a valid relative estimate of the dissociation constants when the metal on-rates are the same for all four sites. This seems a valid assumption since this rate is very close to the diffusion-limited on-rate (Andersson et al., 1982).

cussed above. This is perhaps related to an unexpected orientation of the tyrosines with respect to the Ca^{2+} binding sites in CAM, which is difficult to assess at present in the absence of a crystal structure for the protein.

In conclusion, we feel that, in line with a large body of evidence, our studies provide strong evidence that domains III and IV are the strong Ca^{2+} binding sites in CAM and that they bind in a cooperative fashion. Furthermore, the outcome of these studies emphasizes the notions that the structural relationship between a pair of calcium binding domains needs to be preserved to attain high affinity (Seamon & Kretsinger, 1983) and that α -helices surrounding the calcium binding loop stabilize Ca^{2+} binding (Reid et al., 1981).

Acknowledgments

We are indebted to Dr. Torbjörn Drakenberg for helpful comments and to Dr. Claude B. Klee and A. Aulabaugh for advice on the isolation of the proteolytic peptides.

Registry No. Calcium, 7440-70-2; cadmium-113, 14336-66-4.

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Paper II

Metal Ion and Drug Binding to Proteolytic Fragments of Calmodulin: Proteolytic, Cadmium-113, and Proton Nuclear Magnetic Resonance Studies[†]

Eva Thulin, Anita Andersson, Torbjörn Drakenberg, Sture Forsén, and Hans J. Vogel*

ABSTRACT: Tryptic fragmentation of Ca^{2+} -saturated calmodulin (CaM) takes place mainly at Lys-77; however, proteolysis can occur instead at Arg-74 or Lys-75. This cleavage pattern results in the production of three peptides each of the amino- and carboxy-terminal halves of CaM of slightly different length. A purification scheme for the three carboxy-terminal half-peptides is reported. Proton nuclear magnetic resonance (^1H NMR) studies of peptides comprising the amino- or carboxy-terminal half of CaM reveal the great structural similarity between these two proteolytic fragments and the intact protein. Since this was observed for the apoprotein as well as the Ca^{2+} -saturated protein, this means that the two halves of the protein are independently folded. A comparison of the changes in the ^1H NMR spectra observed for the intact

protein and the fragments upon addition of Ca^{2+} clearly identified sites III and IV as the two high-affinity binding sites. Furthermore, addition of Ca^{2+} or Cd^{2+} induces qualitatively similar changes in the spectra, thus indicating that Cd^{2+} is a reliable replacement for Ca^{2+} in these studies. Subsequent ^{113}Cd NMR studies of trifluoperazine (TFP) binding to tryptic and thrombic fragments of calmodulin revealed the presence of two distinct drug binding sites, one located in the amino-terminal half and one located in the carboxy-terminal half. The spectral changes, induced upon addition of the antipsychotic drug, were similar to those observed upon binding of TFP to intact calmodulin. The strongest TFP binding site is located in the carboxy-terminal half.

Hormonal or nerve impulses can cause the level of cytosolic Ca^{2+} in eukaryotic cells to rise from 10^{-7} to 10^{-5} M. During such an influx, the metal ion binds to regulatory calcium binding proteins like troponin C (TnC)¹ and calmodulin (CaM) which in response undergo large conformational changes. The completion of the amino acid sequence of CaM revealed its high homology to skeletal TnC (Watterson et al., 1980); both proteins contain four calcium binding domains each arranged in a continuous helix-loop-helix sequence, often

called the EF hand (Kretsinger, 1976). TnC is the regulatory calcium binding subunit of the troponin complex that triggers contraction of striated muscles (McCubbin & Kay, 1980). Its soluble analogue CaM exposes hydrophobic regions upon Ca^{2+} binding (LaPorte et al., 1980; Tanaka & Hidaka, 1980; Vogel

¹ Abbreviations: CaM, calmodulin; TnC, troponin C; NMR, nuclear magnetic resonance; DEAE, diethylaminoethyl; TFP, trifluoperazine; SDS, sodium dodecyl sulfate; STI, soybean trypsin inhibitor; 2D, two dimensional; EDTA, ethylenediaminetetraacetic acid. Peptide nomenclature: TR₁C, fragment 1-77; TR₂C, fragment 78-148; TM₁, fragment 1-106; TM₂, fragment 107-148 [see Kuznicki et al. (1981), Wall et al. (1981), and Andersson et al. (1983a)].

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et al., 1983) which are thought to be important for the interaction of CaM with its manifold target proteins [for a review, see Cheung (1980) and Klee & Vanaman (1982)]. One calcium-induced hydrophobic site appears to be located in each half of the molecule (Vogel et al., 1983), and the existence of allosteric interactions between these two sites has been reported (Johnson, 1983). Various hydrophobic drugs, like the antipsychotic drug trifluoperazine (TFP), are powerful inhibitors of the potentiating effects of the calmodulin-calcium complex on its target proteins; the corollary to this, that they compete for the same binding sites on CaM's surface, has been widely accepted (Klee & Vanaman, 1982). Thus, in order to gain an understanding of CaM's structure when bound in complexes, several studies have focused on the study of CaM itself (Klevit et al., 1981; Krebs & Carafoli, 1982) or metal ions bound to CaM (Forsén et al., 1980; Thulin et al., 1980; Andersson et al., 1983b) in drug-protein-metal ion complexes.

Another question which is of imminent importance to an understanding of the capacity of CaM to regulate such a wide spectrum of different metabolic processes is the order in which the sites of apo-CaM are filled by Ca^{2+} . Although it is generally agreed upon that filling of the sites takes place sequentially, rather than randomly (Klee & Vanaman, 1982; Kilhoffer et al., 1983), many conflicting results have been reported. For TnC, unlike CaM, it is firmly established that domains III and IV are the high-affinity binding sites (McCubbin & Kay, 1980). Instrumental for this assignment were studies using proteolytic fragments that had retained the native structural properties of the intact protein (Leavis et al., 1978). Recent cadmium-113 NMR studies utilizing proteolytic fragments of CaM (Walsh et al., 1977; Drabikowski et al., 1977) provided strong evidence for sites III and IV being the positive cooperative high-affinity binding sites for Ca^{2+} (Andersson et al., 1983a). In addition, our affinity chromatographic studies have provided some insight into the location of the Ca^{2+} -induced hydrophobic domains (Vogel et al., 1983a). Here we report on further ^1H and ^{113}Cd NMR studies of these proteolytic fragments which have shed more light on the location of the high-affinity metal ion binding sites and the hydrophobic drug binding sites. In the course of these studies, we also confirmed that tryptic cleavage of calmodulin in the presence of Ca^{2+} mainly takes place at Lys-77 (Walsh et al., 1977). However, we discovered that considerable hydrolysis of other peptide bonds can occur instead.

Experimental Procedures

Materials. Bovine testis calmodulin and its tryptic and thrombin fragments were prepared as described earlier (Andersson et al., 1983a; Vogel et al., 1983a). All other chemicals used were commercially obtained and were of the highest purity available.

Methods. The purity of CaM and its proteolytic fragments was checked by both SDS gel electrophoresis and agarose gel electrophoresis in the presence and absence of 1 mM EDTA or Ca^{2+} . Amino acid compositions and sequence analysis were performed as described earlier (Vogel et al., 1983a). ^{113}Cd NMR experiments were performed on our home-built 6-T NMR spectrometer [for a description, see Drakenberg et al. (1983)] using routine acquisition parameters described elsewhere (Forsén et al., 1980). ^1H NMR spectra of protein dissolved in D_2O were obtained on a Nicolet 360-WB spectrometer as detailed elsewhere (Andersson et al., 1983b). Two-dimensional (2D) proton correlation spectra were acquired with the standard COSY program of the Nicolet NMCFT software by using a 90° - τ - 60° -acquisition pulse sequence with quadrature detection in both dimensions, where

Scheme I

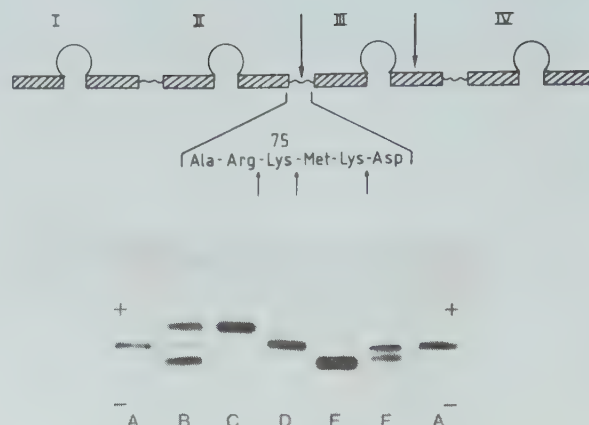


FIGURE 1: Agarose gels (run in the presence of 1 mM EDTA) of CaM and its tryptic fragments: (A) CaM; (B) mixture of $\text{TR}_2\text{Ca,b,c}$; (C) TR_2Ca ; (D) TR_2Cb ; (E) TR_2Cc ; (F) TR_1C . The positions of the cathode and anode are indicated in the figure.

τ was changed in steps of 250 μs corresponding to a spectral width of ± 2000 Hz. Routinely, 96–240 scans were accumulated into 512 blocks of 1024 data points. Resolution enhancement of the two-dimensional spectra was achieved by double-exponential multiplication and symmetrization of the spectra around the diagonal (Nagayama et al., 1979). Gated proton decoupling was used to reduce the intensity of the residual HDO resonance during the two-dimensional experiments.

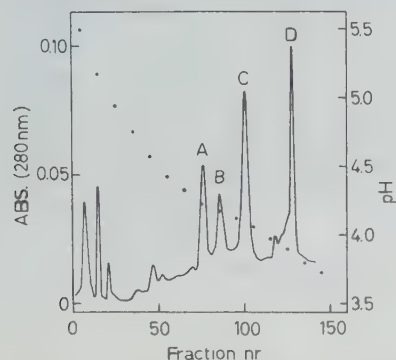
Results

Proteolytic Cleavage of Calmodulin. Scheme I depicts the structure of calmodulin and indicates the reported points of cleavage by thrombin (Arg-106) and trypsin (Lys-77) in the presence of EDTA and Ca^{2+} , respectively (Wall et al., 1981; Walsh et al., 1977). Using amino acid compositions and sequencing studies, we confirmed that thrombin cleavage under these conditions occurs at Arg-106 only (Wall et al., 1981; data not shown). However, agarose gel analysis of the TR_2C fragment of calmodulin² consistently showed the presence of three bands (see Figure 1, lane B), although the starting material CaM moved as one band both on agarose gel electrophoresis (see Figure 1, lane A) and on SDS gel electrophoresis. Thus, it appeared that tryptic cleavage of CaM in the presence of Ca^{2+} results in three TR_2C fragments of slightly different charge. Because some differentiation (but no resolution) of these fragments was obtained upon chromatography on a Sephadex G50 (200 $\times$ 1.4 cm) column, it appeared that the fragments were only slightly different in size. In order to resolve these three fragments, a degradation mix (Andersson et al., 1983a) was immediately applied to a DEAE-Sephacel column (1 $\times$ 34 cm) which was equilibrated in 50 mM sodium acetate, pH 5.5 (adjusted to pH 5.5 with acetic acid). The column was eluted with a combined pH and salt gradient using a buffer containing 50 mM sodium acetate–100 mM acetic acid, pH 3.7 (pH adjusted with HCl). The column profile obtained for this peptide mixture is shown in Figure 2. The peaks that eluted directly from the column contained STI and the trypsin–STI complex; peak D contains undegraded calmodulin as judged by electrophoresis. The

² Our standard proteolysis conditions are such that hardly any peptide TR_1C remains in the proteolysis mixture (Andersson et al., 1983a). Thus, TR_2C can be easily purified by gel filtration from leftover undegraded CaM.

Table I: Amino Acid Sequences Determined for Tryptic Calmodulin Carboxy-Terminal Fragments

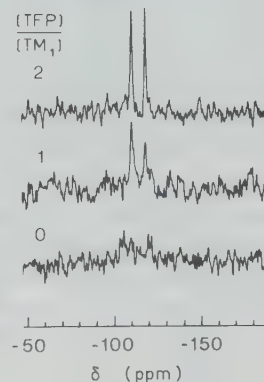
peptide			amino acid sequence														
CaM ^a	A	R	⁷⁵ K	M	K	D	T	⁸⁰ D	S	E	E	E	⁸⁵ I	R	E	A	F
TR ₂ C _a						D	T	D	S	E	E	E	I/K	R	E	A	F
TR ₂ C _b				M	K	D	T	D	S	E	E	E	I	R	E	A	F
TR ₂ C _c			K/L	V/M	K/L	D	T	D	S	E	E	E	I/K	R	E	A	F

^a From Watterson et al. (1980).FIGURE 2: Purification of TR₂C peptides on DEAE-Sephacel using a pH gradient (●). Peaks A, B, C, and D correspond to fragments TR₂C_a, TR₂C_b, TR₂C_c, and CaM, respectively. For further explanation, see the text.

other peaks labeled A, B, and C were separately dialyzed against double-distilled water and lyophilized. Agarose gel electrophoresis showed that every peak contained a pure TR₂C peptide (see Figure 1, lanes C, D, and E for peaks C, B, and A respectively). Subsequent amino acid analysis (data not shown) and sequencing (Table I) studies provided insight into the nature of the three different peptides. Cleavage does not only occur at Lys-77 but also at Arg-74 and Lys-75, thus giving rise to the three fragments of slightly different charge and length. Since TR₁C also runs as three bands on agarose gel electrophoresis (Figure 1, lane F), it is likely that TR₁C is a mixture of the peptides 1-74, 1-75, and 1-77. However, since the relative intensities of the bands in Figure 1F do not correspond to the yields of the TR₂C peptides, it is likely that further proteolysis of fragments can occur.

Cadmium-113 NMR Studies of Proteolytic Fragments. Titration of CaM with ¹¹³Cd²⁺ gives rise to two well-resolved signals in the ¹¹³Cd NMR spectrum (slow exchange on the NMR time scale) that grow parallel in intensity up to 2 equiv of Cd²⁺ per CaM. Subsequent addition of 2 more equiv does not give rise to any detectable resonance; however, upon addition of further equivalents, a signal for free Cd²⁺ ions is observed (Forsén et al., 1980). The nondetectability of the signals for the third and fourth equivalent of ¹¹³Cd²⁺ has been attributed to chemical exchange between free and protein-bound Cd²⁺ ions (Forsén et al., 1980). Since monovalent cations like Na⁺ and K⁺ can bind to the protein (Delville et al., 1980; Haiech et al., 1981), it appeared worthwhile to investigate the effect of salts on the binding of metal ions. However, titration experiments performed in the presence of 0.15 M KClO₄ gave results identical with those discussed above.

We have recently shown that addition of 2 equiv of ¹¹³Cd²⁺ to peptide TR₂C (in fact a mixture of the three TR₂C fragments) gives rise to the same two well-resolved resonances as for CaM (Andersson et al., 1983a). No resonance is observed if 2 equiv of ¹¹³Cd²⁺ is added to fragment TR₁C. Subsequent addition of Ca²⁺ liberates the bound ¹¹³Cd²⁺ ions which results in a broad resonance at -12 ppm for Cd²⁺ (spectra included in the supplementary material; see paragraph at end of paper

FIGURE 3: Increase of signals in the ¹¹³Cd NMR spectrum of Cd²⁺-saturated TM₁ (0.92 mM, pH 7.0) upon TFP titration (5×10^4 acquisitions). The bottom spectrum cannot be directly compared to that reported earlier (Andersson et al., 1983a) since that was obtained at a somewhat lower temperature.

regarding supplementary material). Thus, the two calcium binding sites of CaM that exhibit the intermediate to fast chemical exchange are located in this fragment, consistent with the observation that the two slow exchanging sites are located in fragment TR₂C. In further experiments, we have attempted to visualize the two Cd²⁺ ions bound to TR₁C by trying to reduce the metal ion exchange rate by lowering the temperature. However, a reduction from 23 to 2 °C had no effect on the ¹¹³Cd NMR spectrum of TR₁C. Similar low-temperature spectra were obtained for CaM, TR₂C, TM₁, and TM₂. We found that the broad signals observed for Cd²⁺ bound to TM₁ and TM₂ (Andersson et al., 1983a) sharpened somewhat. In addition, all observable protein-bound ¹¹³Cd²⁺ resonances showed small downfield shifts when the temperature was lowered to 2 °C (varying between 1 and 4.5 ppm). These effects are comparable to those observed for the ¹¹³Cd²⁺ NMR reference signal [0.1 M Cd(ClO₄)₂] which itself shifts 3.5 ppm downfield between 23 and 2 °C.

Effect of Trifluoperazine on the ¹¹³Cd NMR Fragment Spectra. Addition of hydrophobic drugs to CaM results in large changes in the ¹¹³Cd NMR spectra (Forsén et al., 1980; Andersson et al., 1983b). The two most pronounced effects that occur upon binding of 2 equiv of TFP are the following: (1) an upfield shift for the two slow exchange signals; (2) a drastic reduction of the exchange rate for the fast to intermediate exchanging ¹¹³Cd²⁺ ions, so that two additional sharp resonances (<50 Hz) appear in the spectrum.³ Figure 3 shows the effect of the addition of TFP on the ¹¹³Cd NMR spectrum of TM₁ (1-106). Two sharp resonances appear, indicating that the drug binding site responsible for the decrease in the metal ion exchange rate of the two weak metal ion binding sites is

³ Since the on rate of the metal ion for all four Ca²⁺ binding sites is thought to be diffusion limited, a decreasing off rate (approximately equal to the metal ion exchange rate as detected by NMR) indicates an increase in the binding constant. Also, ⁴³Ca NMR studies have provided evidence for a tighter binding of metal ions in the presence of TFP (Thulin et al., 1980).

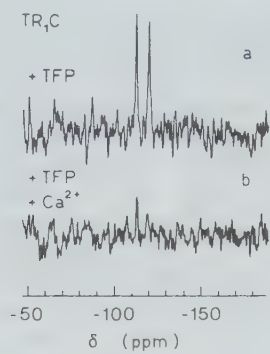


FIGURE 4: ¹¹³Cd NMR spectra of Cd²⁺-saturated TR₁C (0.97 mM, pH 7.5) after addition of (a) 2 equiv of TFP and (b) 3 equiv of TFP and 2 equiv of Ca²⁺ (5 × 10⁴ acquisitions).

Table II: Chemical Shifts Measured for the Protein-Bound ¹¹³Cd Signals in the Absence and Presence of Trifluoperazine

protein	[TFP]: [protein]	chemical shift (ppm)			
		A	C	D	B
CaM	0	-87.5	n.o. ^a	n.o.	-114.2
	2.7	-99.1	-112.9	-115.2	-118.1
TR ₂ C	0	-87.5			-114.2
	3.0	-100.0			-118.0
TR ₁ C	0		n.o.	n.o.	
	3.0		-112.0	-119.5	
TM ₁	0		n.o.	n.o.	-109 ^b
	3.0		-111.5	-119.0	n.o.
TM ₂	0	-95 ^b			
	2.0	-95 ^b			

^a n.o. = not observable due to exchange broadening. ^b Broad resonance (>200 Hz).

located in this peptide and is functioning as in the native protein. The same two resonances are observed with peptide TR₁C after addition of TFP (see Figure 4a). When TFP was added to a sample containing ¹¹³Cd²⁺-loaded TR₂C, the two sharp Cd²⁺ signals shifted as for CaM (see Table II). Also, the broadening observed for the most downfield resonance in the midpoint of the titration of CaM [see Figure 2D in Forsén et al. (1980)] was observed here. It is further noteworthy that when one adds up the spectra for TFP-saturated TR₁C and TR₂C a spectrum closely resembling that of TFP-saturated CaM is obtained (compare Table II). Significantly, addition of TFP to fragment TM₂ (107–148) did not cause a change in the ¹¹³Cd NMR spectrum. We also investigated competition between Ca²⁺ and Cd²⁺ in these TFP-saturated peptides (see, for example, Figure 4). In all instances where ¹¹³Cd NMR signals with chemical shifts between -80 and -120 ppm were observed, these signals disappeared after the addition of an excess of Ca²⁺ ions, and a signal with a chemical shift close to that of free Cd²⁺ ions appeared.

¹H NMR Structural Comparison between CaM and Fragments. Figures 5 and 6 compare the aromatic and the upfield-shifted methyl group regions of the 360-MHz ¹H NMR spectra of apo- and calcium-loaded CaM, TR₁C, and TR₂C as well as the difference spectrum CaM - (TR₁C + TR₂C) which was obtained by computer subtraction. Extensive studies by other groups have provided tentative assignments of various resonances to specific residues for apo-CaM as well as Ca²⁺-CaM (Seamon, 1980; Krebs & Carafoli, 1982; Ikura et al., 1983a,b). A listing of the assignments of the most important resonances is shown in Table III. Proton resonances that are normally most sensitive to changes in the protein's structure are those that are shifted by ring current effects induced by proximal aromatic groups. Such resolved

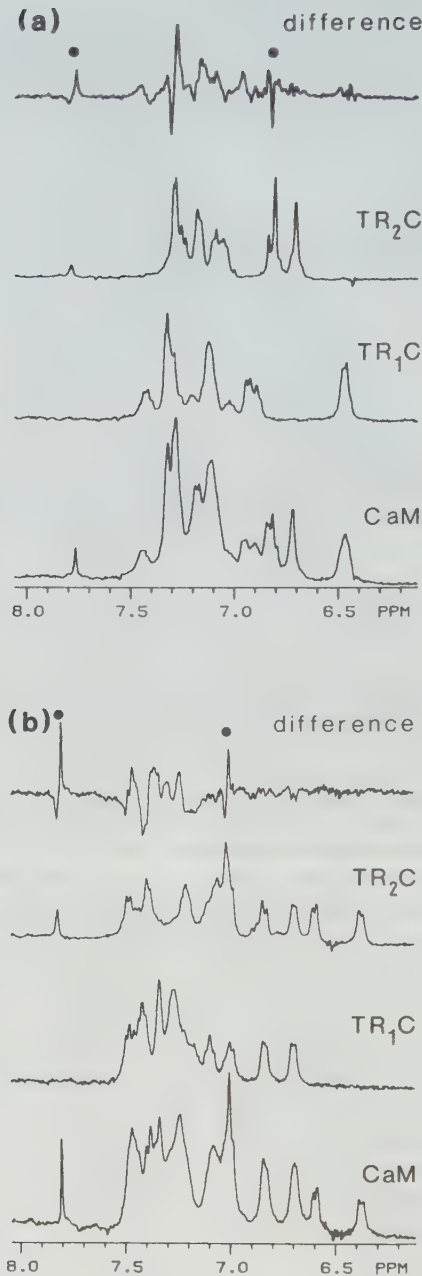


FIGURE 5: Aromatic region of the ¹H NMR spectra of TR₁C, TR₂C (both 1.5 mM, pH 7.5), and CaM (2.0 mM, pH 7.5) and the difference spectra of CaM - (TR₁C + TR₂C): (a) apo forms; (b) Ca²⁺-saturated forms. The solid dots indicate the positions of the His-107 resonances, which are very sensitive to small changes in pH and hence show up in the difference spectra (500 acquisitions).

resonances are found between 6.3 and 6.9 ppm and -0.2 and 0.6 ppm in the spectra. Visual inspection reveals the great similarity for both the apo- and the calcium-saturated CaM in these regions of the spectra (Figures 5 and 6). For example, the two most highfield-shifted phenylalanine resonances in the TR₁C fragment as well as the most highfield phenylalanine and the four tyrosine resonances in the TR₂C fragment all have the same shift as in calmodulin itself and consequently disappear in the difference spectrum (Figure 5a). This indicates that the two halves of the CaM molecule have the same conformation whether they are covalently attached to each other as in calmodulin or physically separated as in the fragments. The same holds for the apoprotein as well as for the Ca²⁺-loaded protein. A similar comparison of the ¹H NMR spectra from the two thrombic fragments with that of intact calmodulin shows that these fragments do not have the

Table III: Assignments of Proton Resonances of Calmodulin and Its Fragments^a

residue	proton	chemical shift (ppm) ^d		proteolytic fragment	conformational exchange rate
		Ca ²⁺ free	Ca ²⁺ loaded		
His-107	δ	7.85 ^b	7.79 ^b	TR ₂ C	slow
	ε	6.82 ^b	7.00 ^b	TR ₂ C	
Tyr-99	δ	6.86	6.82	TR ₂ C	slow
	ε	7.14	6.98	TR ₂ C	
Tyr-138	δ	6.75	6.36	TR ₂ C	slow
	ε	6.75	6.58	TR ₂ C	
Tml-115 ^c	ε	3.13	3.11	TR ₂ C	slow
Phe-A	δ	6.47	6.64	TR ₁ C	fast
Phe-B	δ	6.47	6.78	TR ₁ C	fast
Val-A	γ	0.52		TR ₁ C	
Val-B	γ	0.43		TR ₂ C	slow
Ile-A	γ	-0.14		TR ₁ C	fast
	δ	0.33		TR ₁ C	fast

^a Assignments from Seamon (1980), Krebs & Carafoli (1982), and Ikura et al. (1983a,b). ^b pH dependent. ^c Tml is trimethyllysine.

^d Our observation for both fragments and CaM.

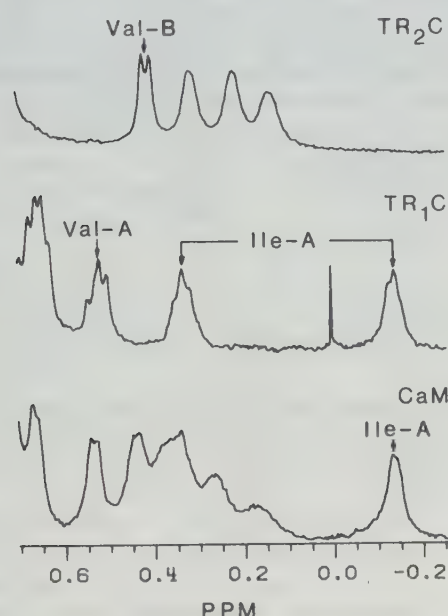


FIGURE 6: Most upfield part of the ¹H NMR spectra of TR₁C, TR₂C, and CaM in the apo form. (See Figure 5 for other conditions. Spectra of the Ca²⁺ form are included in the supplementary material.)

same conformation as the intact protein (data not shown).

A further test for the similarity of the structure of the fragments as compared to the intact protein can be obtained from 2D NMR. In this way, not only the similarity of in-

dividual chemical shifts but also correlation signals from resonances connected by spin couplings can be checked. Figure 7 shows the contour plots for the COSY 2D NMR spectra of calmodulin and its two tryptic fragments. Such a contour plot contains the normal one-dimensional NMR spectrum (viewed from above) along the main diagonal; spin-spin couplings are revealed as off-diagonal peaks connecting the two coupled resonances on the diagonal. It is obvious from Figure 7 that all correlation signals observed in the calmodulin spectrum can also be found in either of the spectra from the fragments. Some extra correlation signals can be observed in the 2D spectra of the fragments, which were not detectable in the 2D spectrum from calmodulin, most probably caused by the fact that the resonances for the lower molecular weight fragments tend to be somewhat sharper and thus give rise to larger cross-peaks.

From a comparison of the spectra obtained for the two tryptic fragments and CaM (Figures 5 and 6), one can decide what resonance in the ¹H NMR spectrum of CaM resides in what half of the protein. We have listed in Table III in which fragment the corresponding resonances were found. As expected, the resonances for tyrosines, histidine-107, and trimethyllysine-115 appear in fragment TR₂C. However, the tentative assignment of Phe-A, -B, and -Z to residues 89, 92, and 141 (Ikura et al., 1983a,b) appears at error since Phe-A and -B are found in the TR₁C fragment. Moreover, Val-A and Ile-A, that are known to be located close to Phe-A and -B, are also in the amino-terminal half of CaM, whereas Val-B is part of the carboxy-terminal half.

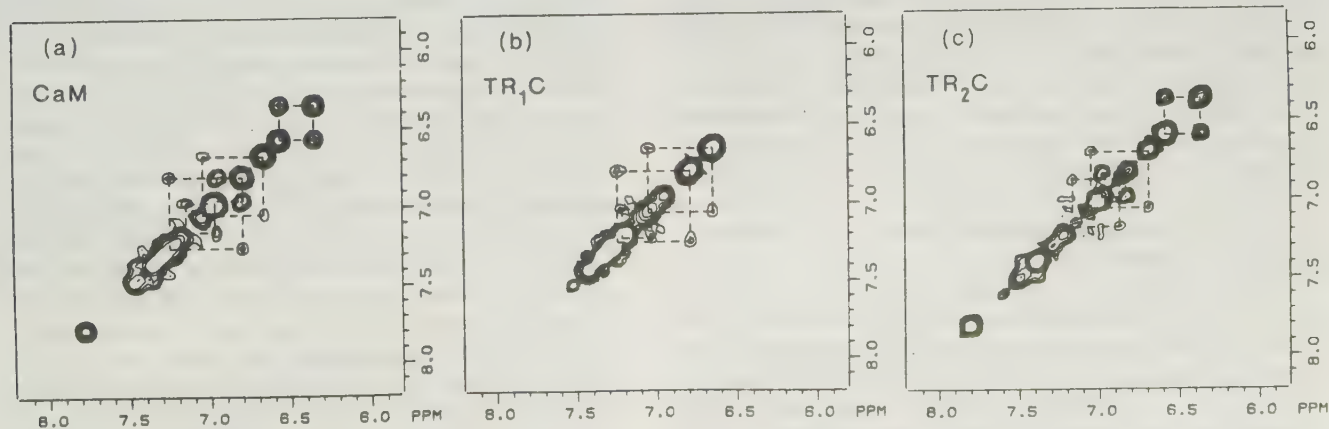


FIGURE 7: Aromatic part of the 2D COSY ¹H NMR spectra of CaM and its tryptic fragments: (a) 2.0 mM CaM, 8.0 mM Ca²⁺, pH 7.5, 240 scans; (b) 1.8 mM TR₁C, 3.6 mM Ca²⁺, pH 7.6, 96 scans; (c) 1.7 mM TR₂C, 3.5 mM Ca²⁺, pH 7.5, 128 scans. For other conditions see Experimental Procedures.

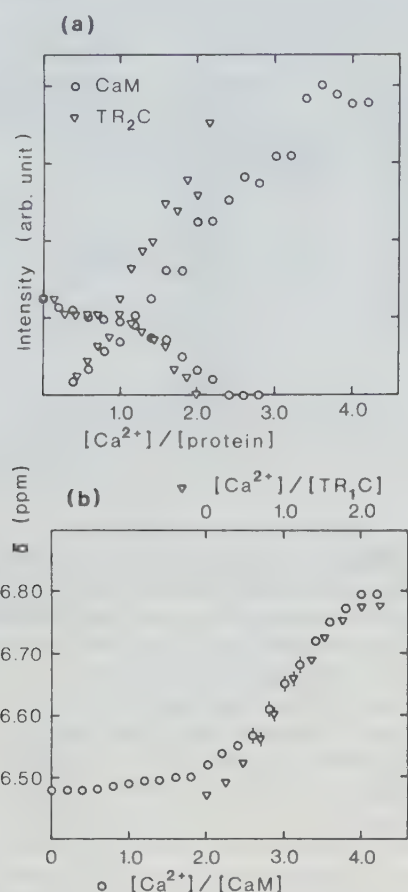


FIGURE 8: Changes in the 1H NMR spectra of CaM and its fragments upon Ca^{2+} addition: (a) intensity of the His-107 resonance in the spectrum of TR₂C (1.5 mM, pH 7.5) or CaM; (b) chemical shift of one upfield-shifted phenylalanine in the spectrum of TR₁C (1.5 mM, pH 7.5) or CaM (2.0 mM, pH 7.5).

Ca^{2+} -Induced Conformational Changes in the Tryptic Fragments. Figure 8a shows the intensities of some resonances in the 1H NMR spectra of CaM and TR₂C as a function of Ca^{2+} saturation. Three resonances from aromatic amino acids can easily be followed as they are well resolved from other resonances in the aromatic region for both the intact protein and the fragments. These resonances are His-107 at 7.85 ppm in the metal-free state and at 7.79 ppm in the Ca^{2+} -loaded state and Tyr-138 at 6.36 ppm in the calcium-saturated state. Since the conformational change due to Ca^{2+} binding in both CaM and TR₂C is in slow exchange (on the proton NMR time scale), a decrease in the intensity of the signals from the apoproteins and an increase in the intensities of the signals from the calcium-saturated proteins can be observed [see Seamon (1980) and Ikura et al. (1983b)]. These changes are observed to occur in an almost identical way for CaM and TR₂C as can be seen from Figure 8a. As expected, the resonances from trimethyllysine-115 (3.13 ppm in the apoprotein and 3.11 ppm when calcium saturated) and tyrosine-138 (and Tyr-99) go through a similar variation as the His-107 resonance (see Table III).

Figure 8b shows the chemical shifts of one of the two phenylalanine resonances in the 1H NMR spectra of CaM and TR₁C as a function of added calcium ions. In the spectrum of TR₁C, these particular resonances are well resolved from other aromatic resonances, while in the spectrum of CaM, overlap with other resonances takes place during the latter half of the Ca^{2+} titration. For CaM, no alteration of the chemical shifts of these signals could be observed up to a Ca^{2+} :CaM

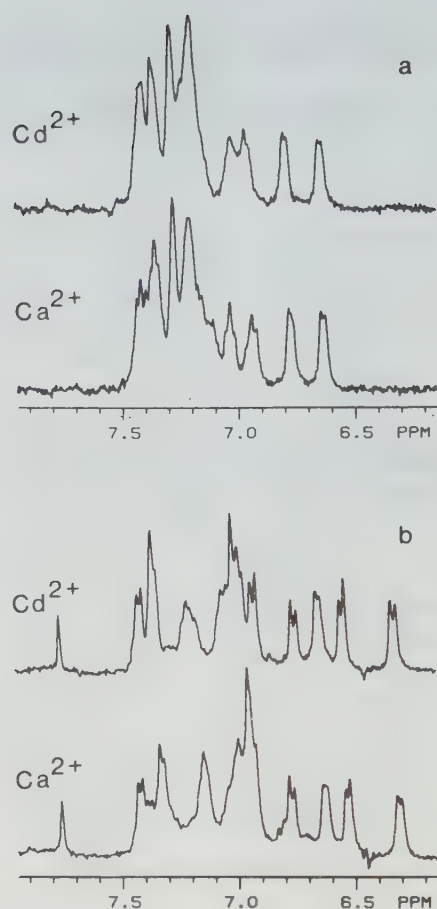


FIGURE 9: Aromatic regions of the 1H NMR spectra of the tryptic fragments. The Ca^{2+} - and the Cd^{2+} -saturated forms are shown: (a) 1.5 mM TR₁C, pH 7.5; (b) 1.5 mM TR₂C, pH 7.5 (500 scans). For spectra of the apo fragments, see Figure 5.

ratio of 2:1.⁴ After this ratio, a continuous change of the chemical shifts can be observed until the final shifts of 6.64 and 6.78 ppm are reached for a Ca^{2+} :CaM ratio of 4:1 (CaM-Ca₄) (Table III). This change is almost identical with the variation in the chemical shifts observed for TR₁C when the Ca^{2+} :TR₁C ratio is varied from 0:1 to 2:1 (see Figure 8b).

Comparison of the Effects of Ca^{2+} and Cd^{2+} on the 1H NMR Spectra of the Tryptic Fragments. Figure 9 shows the aromatic part of the 1H NMR spectra of Ca^{2+} - and Cd^{2+} -saturated TR₁C and TR₂C fragments. In the 1H NMR spectrum of TR₁C, the two highfield-shifted phenylalanine resonances (Phe-A and -B) at 6.47 ppm as well as two others (Phe-C and -D) around 6.9 ppm shift downfield and split into two resonances as the metal ion content is increased. Although the same sequence of events is observed during the calcium as well as the cadmium titration, slight differences of the chemical shifts are measured for the Phe-C and -D resonances (6.94 and 7.04 ppm in TR₁C-Ca₂ and 6.95 and 7.02 ppm in TR₂C-Cd₂). Also in the region of the unshifted phenylalanine resonances, particularly at 7.5 ppm, some minor differences can be observed. A broadening of the highfield-shifted phenylalanine signals can be seen in the course of the titration. The effect is most pronounced in the case of calcium binding.

⁴ Ikura et al. (1983a,b) indicate that Phe-A is affected by both the slow and the fast exchange process, and thus changes during titration between zero and four Ca^{2+} ions, and that Phe-B is affected by the slow exchange process only. However, our calcium titrations on CaM as well as its tryptic fragments do not show this behavior. In our hands, both resonances are only affected by the fast exchange process, which occurs on binding of the third and fourth Ca^{2+} ions to CaM.

This broadening can be used to calculate the lifetime of the conformation induced by calcium or cadmium ion binding to TR₁C. Using $\tau = 2\Delta\nu_{1/2}/(\pi\delta\nu^2)$, where $\Delta\nu_{1/2}$ is the maximum broadening of the line and $\delta\nu$ the chemical shift difference between the two exchanging species, we have calculated the lifetime, τ , to be ca. 0.4 and ca. 0.8 ms for TR₁C–Cd₂ and TR₁C–Ca₂, respectively. In other regions of the proton NMR spectrum of TR₁C, similar effects can be found as in the aromatic region. For example, the highfield Ile-A resonance at –0.14 ppm broadens when Ca²⁺ or Cd²⁺ is added and shifts downfield. However, it should be indicated here that the proton NMR spectra of the Ca²⁺- and Cd²⁺-loaded TR₁C are not exactly identical in the methyl region but display small differences in chemical shifts.

As can be seen in Figure 9, the Ca²⁺- and Cd²⁺-saturated fragment TR₂C's give rise to very similar proton NMR spectra. However, as for the TR₁C fragment, there are some minor differences; for example, the Tyr-99 resonances appear at 6.82 and 6.98 ppm and at 6.79 and 6.96 ppm in the Ca²⁺- and Cd²⁺-loaded forms, respectively. Furthermore, a broadening of the signals in the proton NMR spectra during the cadmium titration is observed, which was not seen in the calcium titration. This broadening corresponds to a lifetime of the TR₂C–Cd₂ complex of ca. 20 ms. The lack of broadening in the calcium titration indicates that the lifetime of the TR₂C–Ca₂ complex is long, $\tau > 0.5$ s.

Discussion

Proteolytic Fragmentation. Since Ca²⁺ binding to CaM causes large conformational changes, proteolytic studies of CaM performed in the presence of either EDTA or Ca²⁺ have given widely different results. Walsh et al. (1977) have provided evidence that proteolytic cleavage of CaM in the presence of Ca²⁺ occurs at Lys-77. The results presented in this paper indicate that indeed ~55% of the hydrolysis products formed is the peptide 78–148. However, a considerable degree of cleavage at Arg-74 (~25%) and Lys-75 (~20%) was observed as well, as the determined amino acid compositions and amino acid sequences (Table I) indicate. Since not only TR₂C but also fragment TR₁C runs as the three bands of different charge, it is clear that proteolytic attack can occur at either one of these three positions, rather than that the main product 78–148 arises from further degradation of the 75–148 or the 76–148 peptide. Thus, this implies surface location and considerable flexibility for these three residues in the presence of Ca²⁺. These results are in excellent agreement with chemical modification studies showing that carboxymethylation of the methionine residues 71, 72, and 76 was facilitated by the presence of Ca²⁺ (Walsh & Stevens, 1978). Since this modification prevents the productive interaction of CaM with cyclic phosphodiesterase, it appears that the Ca²⁺-exposed flexible hinge region connecting CaM's D and E helices as well as helix D plays an important role in the interaction of CaM with its target proteins.

Cadmium-113 NMR Studies of Proteolytic Fragments of Calmodulin. In order for ¹¹³Cd NMR of proteolytic fragments of calmodulin to be a valid approach for determining the calcium binding properties of the intact protein, the following three conditions have to be fulfilled: (1) the conformation of the fragments has to closely resemble that of the intact protein; (2) the conformational changes induced by binding of metal ions have to be the same for the fragments and CaM; (3) Cd²⁺ has to act similarly to Ca²⁺. These three conditions are discussed in the following sections.

(1) Our ¹H NMR data presented in Figures 5–7 provide strong evidence for the idea that the NMR spectrum of intact

CaM is closely resembled by the sum of the spectra of its two tryptic fragments. Particularly the observation that all the ring current shifted resonances, which are the most sensitive ¹H NMR parameters of a protein's folding, appear at identical chemical shifts implies that both halves of calmodulin must be independently folded. Although binding of Ca²⁺ causes conformational changes resulting in large changes in the pattern of ring current shifted resonances, the agreement between the sum of the tryptic spectra and that of CaM, for both the apo- and the Ca²⁺-loaded forms of the protein, is very good. Studies using a series of other spectroscopic methods are in good agreement with our ¹H NMR data since they also indicate the structural similarity between the conformations of the two independent halves of the protein and the intact CaM (Drabikowski et al., 1982). Thus results obtained with the tryptic (but not the thrombic) fragments can be extrapolated to intact CaM.

(2) With regard to the second question, our ¹H NMR data, as presented in Figure 8, for example, clearly indicate that binding of Ca²⁺ causes the same sequence of events when binding to CaM or to its tryptic fragments (see also the section below on the sequence of filling). Titration studies using other spectroscopic techniques also provided evidence for the fact that the same conformational changes are obtained for the tryptic fragments and intact CaM (Drabikowski et al., 1982).

(3) The final requirement (that Cd²⁺ has to act similarly to Ca²⁺) has been studied in various ways. Studies in our laboratory on proteins homologous to CaM have shown that Cd²⁺ is a reliable replacement for Ca²⁺ [see Vogel et al. (1983b) and Forsén et al. (1983)]. Since both Cd²⁺ and Ca²⁺ induce the same change in electrophoretic mobility (Andersson et al., 1983a), the conformations of Cd²⁺- and Ca²⁺-saturated CaM and fragments appear to be similar. The most demanding test for Cd²⁺ as a reliable Ca²⁺ substitution probe is provided, however, by following the changes in the ¹H NMR spectra for both Ca²⁺ and Cd²⁺ in titration experiments. For intact CaM, we and Klevitt (1981) have shown that addition of each metal ion causes almost identical changes in the ¹H NMR spectra; only minor differences are observed in the metal ion saturated CaM (Forsén et al., 1983). Figure 9 compares the spectra for the tryptic fragments obtained in the presence of Ca²⁺ or Cd²⁺. The spectra are very similar; only minor differences can be detected. During the titration from 0 to 2 equiv of Ca²⁺ and Cd²⁺, the same sequence of events take place (i.e., resonances shift the same directions and approximately at the same rates in both titrations). Thus, on the whole, these data are all in support of the idea that Cd²⁺ is a reliable substitute for Ca²⁺, since it induces conformational changes in the same order and to the same extent as calcium.

Assignment of Strong and Weak Cation Binding Sites. The ¹¹³Cd NMR spectrum of TR₂C resembles CaM–Cd₂ (Andersson et al., 1983a) whereas the ¹¹³Cd NMR spectrum of Cd²⁺-loaded TR₁C shows no resonances. This resembles what happens when the third and fourth equivalent of Cd²⁺ is added to CaM–Cd₂ to give CaM–Cd₄. Thus, these data indicate that domains III and IV are the high-affinity metal ion binding sites and domains I and II are the low-affinity sites. Further evidence for this sequence of filling is provided by the ¹H NMR studies of the proteolytic fragments. In ¹H NMR studies of intact CaM, resonances could be divided in three groups depending on their Ca²⁺ titration behavior: group I, slow conformational exchange on the ¹H NMR time scale (one signal increases and concomitantly another decreases); group II, fast exchange (a signal shifts gradually from one chemical shift position to another); group III, resonances affected by

both processes (Seamon, 1980; Ikura et al., 1983b). The results presented here (see Figure 8 and Table III) indicate that all the resonances sensing the slow exchange processes are located in the carboxy-terminal fragment, whereas the resonances associated with the fast exchange process are located in the amino-terminal fragment. Thus, these ^1H NMR studies on the proteolytic fragments also show that the carboxy-terminal half contains the two high-affinity sites, where Ca^{2+} binding gives rise to slowly exchanging conformations, and that domains I and II are the low affinity sites. Although the same pattern had been previously inferred from ^1H NMR studies of intact CaM (Seamon, 1980; Ikura et al., 1983b), one can never be sure that binding to half of the molecule does not cause large conformational changes that are transmitted to the other side of the molecule. However, since the results with the proteolytic fragments and CaM reported here are similar, we can conclude that the majority of the conformational rearrangements detected in the ^1H NMR spectra must be localized to the half of the protein containing the calcium binding sites involved in Ca^{2+} binding.

As discussed elsewhere (Andersson et al., 1983a), other researchers have suggested other schemes for the ordered filling of CaM's metal ion binding sites. However, the majority of these studies used trivalent lanthanides (Kilhoffer et al., 1983; Wang et al., 1982; Wallace et al., 1982; Krebs & Carafoli, 1982) or monovalent cations (Delville et al., 1980; Haiech et al., 1981) to probe the calcium binding sites of CaM. Although lanthanides do support CaM activity (Wallace et al., 1982; Klee & Vanaman, 1982), no evidence has been presented to date that they have the same sequence of filling as Ca^{2+} . In fact, the conformational changes observed upon titration of CaM with La^{3+} that can be followed by ^1H NMR indicate a different sequence of filling for this cation than for Ca^{2+} and Cd^{2+} (W. Niemczura, unpublished results). Also, the studies using monovalent cations rely on the assumption that these cations and Ca^{2+} compete for the same metal ion binding sites (Delville et al., 1980; Haiech et al., 1981). This assumption is not necessarily correct. β -Parvalbumins, for example, contain a monovalent cation binding site close to one of the Ca^{2+} -EF hand sites, whose metal ion binding properties are strongly influenced by occupation of the EF hand site (Cavé et al., 1982). Similar sites could exist in CaM or TnC and could explain the reported observations.

It should be noted that the assignment of domains III and IV as the strong binding sites would make calmodulin in this respect similar to skeletal TnC (Leavis et al., 1978; Sin et al., 1978) and cardiac TnC (Hincke et al., 1981; Teleman et al., 1983). This order of binding has also been predicted on the basis of the amino acid similarities between TnC and CaM (Reid & Hodges, 1980; Klee & Vanaman, 1982).

Interaction of Calmodulin and Drugs. Since binding of TFP to TR_1C and TR_2C causes specific changes in the ^{113}Cd NMR spectra of these fragments (Figures 3 and 4, Table II) that are virtually indistinguishable from those observed upon titration of intact CaM with TFP (Forsén et al., 1980), it appears that the two binding sites for the TFP molecules are still largely intact. Thus, one such calcium-induced binding site is located in the amino-terminal half of the protein and the other in the carboxy-terminal half of the protein (Vogel et al., 1983). The location of the latter site is most probably not solely on the carboxy-terminal helix of CaM as had been suggested (Klevit et al., 1981), since no changes in the ^{113}Cd NMR spectrum of fragment TM_2 were observed upon TFP addition. In fact, evidence has been provided for its location in the fragment 77-124 (Head et al., 1982), although location

in the stretch 77-106 appears less likely (Vogel et al., 1983a). From a comparison of the changes in the ^{113}Cd NMR spectra of the tryptic fragments (Figures 3 and 4) and intact CaM [see Figure 2 in Forsén et al. (1980)], one can in fact conclude that the TFP binding site that is located on the TR_2C fragment has a higher affinity for this drug than the binding site located in the amino-terminal half: upon binding of the first TFP molecule to CaM, the changes in the CaM spectrum resemble those observed for TR_2C , whereas the changes observed with TR_1C resemble mostly those observed upon binding of the second TFP molecule to CaM.

Calmodulin Action. Although all the studies discussed above indicate that the two tryptic fragments bear a remarkable resemblance to intact CaM, it would be too simple to suggest that CaM is nothing more than the sum of these two parts. The fact that higher levels of TFP than normally needed for CaM were required in this study to saturate the TR_1C fragment indicates a weaker binding for this hydrophobic drug. This may be caused by the fact that the allosteric interactions between the two drug binding sites (Johnson, 1983) are lost in the fragments. The fact that such interactions exist, plus the existence of a group of proton resonances sensitive to the filling of all four Ca^{2+} binding sites (Seamon, 1980; Ikura, 1983a), indicates that the two independently folded halves of calmodulin do interact with one another. Another point of interest is that proteolytic fragments in general have been disappointingly poor in imitating CaM's effects on the activity of its target enzymes and proteins (Walsh et al., 1977; Kuznicki et al., 1981; Wall et al., 1981; Schreiber et al., 1981). These observations appear to indicate that binding of both Ca^{2+} -exposed "drug" binding domains to the majority of the target enzymes is a necessity to bring about the changes in activity. Klee & Vanaman (1982) pointed out that it is unfortunate that in most of the fragment studies only activation and not the binding of fragments has been determined. If binding of both interaction domains is a requirement for activation, binding of a fragment, with only one interaction site, need not be accompanied by activation. Thus, as a working hypothesis for further experiments, it seems that calmodulin bears a closer resemblance to skeletal TnC than previously thought (Kilhoffer et al., 1983). Positive cooperative binding of the first two Ca^{2+} ions to domains III and IV induces the exposure of the carboxy-terminal interaction site; this is thought to be sufficient to establish binding between CaM and a target enzyme but not necessarily activation. Likewise, the interaction between TnC and the other troponin subunits is fully dependent on the occupancy of its two high-affinity metal binding sites by Ca^{2+} or Mg^{2+} (Cox et al., 1981). As a second step, saturation of CaM's weaker binding sites I and II would expose the second interaction site,⁵ which would confer the regulatory action on the activity. In parallel, for TnC, occupation of these sites is considered to be the main regulatory mechanism for muscle contraction (Robertson et al., 1981). It is clear that this mode of CaM's functioning requires further study. However, it does appear to provide a rationale for the intracellular appearance of carboxy-terminal fragments of CaM (Schreiber et al., 1981). These can act as regulators since they can bind to target enzymes and thus block CaM's

⁵ This model would fit with the fact that mostly three or four bound Ca^{2+} ions per CaM are required for activation. Certain target enzymes require, however, only two bound Ca^{2+} ions (Klee & Vanaman, 1982). For these, it appears either that binding of the second activation domain is not necessary (a notion not supported by the results obtained by the activation experiments with the proteolytic fragments) or that such binding can take place, without Ca^{2+} binding to sites I and II.

activating interactions, as was recently demonstrated (Newton & Klee, 1983).

Acknowledgments

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Supplementary Material Available

Two figures showing ¹¹³Cd NMR spectra of Cd²⁺ binding to TR₁C as well as a comparison of the upfield-shifted methyl region ¹H NMR spectra of Ca²⁺-saturated CaM, TR₁C, and TR₂C (2 pages). Ordering information is given on any current masthead page.

Registry No. TFP, 117-89-5; Ca, 7440-70-2; Cd, 7440-43-9; ¹¹³Cd, 14336-66-4.

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Paper III

Kinetics of calcium dissociation from calmodulin and its tryptic fragments

A stopped-flow fluorescence study using Quin 2 reveals a two-domain structure

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The kinetics of calcium dissociation from bovine testis calmodulin and its tryptic fragments have been studied by fluorescence stopped-flow methods, using the calcium indicator Quin 2. Two distinct rate processes, each corresponding to the release of two calcium ions are resolved for calmodulin at both low and high ionic strength. The effect of 0.1 M KCl is to accelerate the slow process from $9.1 \pm 1.5 \text{ s}^{-1}$ to $24 \pm 6.0 \text{ s}^{-1}$ and to reduce the rate of the fast process from 650 s^{-1} to $240 \pm 50 \text{ s}^{-1}$ at 25°C. In the presence of 0.1 M KCl it was possible to determine activation parameters for the fast process: $\Delta H^\ddagger = 41 \pm 5 \text{ kJ mol}^{-1}$ and $\Delta S^\ddagger = -63 \pm 17 \text{ J K}^{-1} \text{ mol}^{-1}$. These values are in good agreement with those obtained by ^{43}Ca NMR.

Studies of the tryptic fragments TR₁C and TR₂C, comprising the N-terminal or C-terminal half of calmodulin, clearly identified Ca^{2+} -binding sites I and II as the low-affinity (rapidly dissociating) sites and sites III and IV as the high-affinity (slowly dissociating) sites. The kinetic properties of the two proteolytic fragments are closely similar to the fast and slowly dissociating sites of native calmodulin, supporting the idea that calmodulin is constructed from two largely independent domains.

The presence of the calmodulin antagonist trifluoperazine markedly decreased the Ca^{2+} dissociation rates from calmodulin. One of the two high-affinity trifluoperazine-binding sites was found to be located on the N-terminal half and the other on the C-terminal half of calmodulin. The affinity of the C-terminal site is at least one order of magnitude greater.

Calmodulin (CaM) is a multifunctional calcium-binding regulator protein found in all eukaryotic cells [1, 2]. The protein belongs to the Troponin C superfamily of high-affinity calcium-binding proteins. The primary sequence consists of four homologous domains (numbered I–IV from the N-terminal), each of which contains an α -helix–calcium binding loop–helix region often referred to as the EF-hand [3]. Upon binding calcium ions the protein undergoes conformational changes that expose sites on the surface allowing it to interact with a number of enzymes and other proteins (for reviews see [1, 4–6]). Several lines of evidence suggest that the interaction between CaM and its target proteins to a large extent depends on hydrophobic interactions. A number of hydrophobic substances, among which are found many drugs in current use [7–9], bind to CaM in a Ca^{2+} -dependent manner. Many of these drugs, for example trifluoperazine (TFP), also inhibit the CaM regulation of target enzymes. It has therefore been assumed that the inhibitory drugs and the target proteins interact with common surface regions of CaM.

The regulatory effect of CaM is intimately linked with its Ca^{2+} -binding ability. Questions of interest concern the order and rate by which the four calcium binding sites are filled with Ca^{2+} . Experimental studies employing a variety of biophys-

ical methods — absorption spectroscopy, circular dichroism, fluorescence spectroscopy, ^1H , ^{43}Ca and ^{113}Cd NMR (for a recent review cf. [10]) — indicate that calcium-binding sites III and IV are filled first. It has recently been suggested, however, that at higher ionic strengths this overall picture may not be valid [11]. The rate of Ca^{2+} binding to CaM is too fast to be measured directly. We have recently shown that the rate of release of Ca^{2+} ions from calcium-loaded CaM can be conveniently studied by stopped-flow techniques using the fluorescent Ca^{2+} chelator Quin 2 [12, 13]. In the present work this kinetic study is considerably extended. The number of Ca^{2+} ions released in two distinct rate processes has been determined. Effects of ionic strength on the rates are investigated. In studies of the solution properties of CaM, one line of research that has proven to be particularly useful is the employment of proteolytic fragments. We have therefore also investigated the rate of release of Ca^{2+} ions from the tryptic fragments TR₁C and TR₂C that correspond closely to the N-terminal and C-terminal half of CaM. The combined kinetic results from CaM and the tryptic fragments lend support to the notion that CaM is made up of two independent domains that may be identified with these fragments.

Finally we have also investigated the effects of the hydrophobic drug TFP on the Ca^{2+} dissociation rates of CaM and its tryptic fragments.

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Abbreviations. CaM, calmodulin; Ca₄-CaM, calcium-saturated calmodulin; Quin 2, (2-[2-bis[carboxymethyl]amino-5-methyl-phenoxy)methyl]-6-methoxy-8-bis[carboxymethyl]amino-quinoline; TFP, trifluoperazine; SDS, sodium dodecyl sulfate; TR₁C, peptide fragment 1–77; TR₂C, peptide fragment 78–148; EEDTA, [(2,2'-oxydiethylene)dinitrilo]tetra-acetic acid.

MATERIALS AND METHODS

Bovine testis calmodulin and its tryptic fragments were prepared as described earlier [14, 15]. The purity of CaM and its proteolytic fragments was checked by both SDS and

Table 1. Kinetic and thermodynamic parameters for the dissociation of Ca^{2+} from CaM in the presence/absence of 0.1 M KCl at 25°C
Values for NMR are from [19]. n.d., not determined. Values given in parenthesis are calculated SD

Phase	Method	k_{obs}	Amplitude	$\Delta H^\#$	$\Delta S^\#$
		s^{-1}	%	$\text{kJ} \cdot \text{mol}^{-1}$	$\text{J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$
Fast	NMR	1000 (100)	—	43.1	−41.8
	SF ^a	650	n.d.	n.d.	n.d.
	SF (+ KCl)	240 (50)	47 (3)	41.0 (5)	−62.7 (16.7)
Slow	NMR	30.0	—	51.4	−41.8
	SF ^a	9.1 (1.5)	n.d.	58.9 (10.5)	−29.3 (29.3)
	SF (+ KCl)	24.0 (6.0)	53 (3)	63.5 (5.4)	− 8.4 (21)

^a Values for these stopped-flow experiments are from [12]; the value for k_{obs} (fast phase) of 650 s^{-1} was estimated from data at lower temperatures.

agarose gel electrophoresis in the presence and absence of 1 mM EDTA or Ca^{2+} . A small contamination (< 5%) of each fragment with the other fragment cannot be excluded due to partly overlapping bands [16] on both SDS and agarose gel electrophoresis. Ca^{2+} -free proteins were prepared by passage through a Chelex-100 column; the residual metal ion was determined by atomic absorption spectroscopy (< 0.05 Ca^{2+} /protein). The absence of EDTA in the protein solutions was checked by ^1H NMR at 360 MHz. Quin 2 (free acid) was obtained from Lancaster Synthesis Ltd, Morecambe, UK.

All other chemicals used were of analytical grade and were obtained from local suppliers. All experiments were performed in a buffer consisting of 20 mM Pipes/KOH, pH 7.0. Doubly distilled water was used throughout and the solutions were stored in plastic containers.

Stopped flow experiments were performed using the system described in detail elsewhere [17]. Quin 2 fluorescence was excited with the mercury arc line at 366 nm. Fluorescence emission was measured at wavelengths greater than 475 nm, where Quin 2 fluorescence decreases upon binding to Ca^{2+} [18]. Data was collected on a DL 905 transient recorder using the split timebase facility and pre-triggering. After transfer to a PDP 11/23 computer, the data was analysed by standard non-linear least-squares methods. Amplitudes reported here have been corrected for losses during the instrumental deadtime (1.1 ms), and are quoted throughout as percentages of the total reaction amplitude.

The dissociation of Ca^{2+} from the Ca-Quin 2 complex was measured by reacting 100 μM Ca^{2+} plus 50 μM Quin 2 with 10 mM EDTA under stopped-flow conditions. The measured dissociation rates varied from 32.3 s^{-1} at 13.5°C to 62.3 s^{-1} at 30.9°C. We have previously reported that the association rate constant for the reaction between Ca^{2+} and Quin 2 is too fast to be measured directly with the stopped-flow technique. Nevertheless, for a simple bimolecular reaction with $K_d = 8 \times 10^{-8}$ M, we calculate $k_{\text{on}} = 4 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ from the value of the dissociation rate measured at 13.5°C. Under pseudo first-order conditions ([Quin 2] = 100 μM ; [Ca^{2+}] < 10 μM) the observed forward rate for calcium chelation will be

$$k_{\text{obs}}^{\text{forward}} = k_{\text{on}}[\text{Quin 2}] + k_{\text{off}} \approx 4 \times 10^4 \text{ s}^{-1}.$$

Therefore, the reaction of Ca^{2+} with Quin 2 will be very much faster than the dissociation of Ca^{2+} from CaM and its use as a kinetic indicator is thus fully justified [12].

In a typical experiment, the Ca_4 -CaM complex is mixed with an excess of Quin 2 under stopped-flow conditions to yield the following concentrations, after mixing: calmodulin

6.25–7.50 μM , Ca^{2+} 62.5 μM and Quin 2 100 μM . Experiments with the fragments were performed under the same conditions. For experiments involving KCl, all reagents were made 0.1 M in KCl; for experiments involving TFP this was added to the Ca_4 -CaM complex prior to the stopped-flow experiments. Concentrations of TFP quoted in the text refer to the concentration after mixing.

RESULTS

Whole calmodulin in the presence/absence of KCl

In previous work [12] we measured kinetic parameters for the Quin-2-induced dissociation of Ca^{2+} from the Ca_4 -CaM complex at three temperatures in the range 11–28°C. Two phases were detected and activation parameters were determined for the slow phase. In the present work this reaction was further studied at a single temperature (11°C), in order to determine the relative amplitudes of the two phases, with the following results; $k_{\text{obs}}^{\text{f}} = 310 \pm 80 \text{ s}^{-1}$, $A^{\text{f}} = 41.8 \pm 6\%$, $k_{\text{obs}}^{\text{s}} = 3.6 \pm 1.2 \text{ s}^{-1}$ and $A^{\text{s}} = 58.2 \pm 6\%$. The superscripts f and s refer to fast and slow phase respectively. The observed rate constants agree well with those obtained previously [12] and it is clear that each phase accounts for approximately half of the total amplitude.

Kinetic parameters for the Quin-2-induced dissociation of Ca^{2+} from the Ca_4 -CaM complex in the presence of 0.1 M KCl have been measured at eight temperatures in the range 11.3–30.1°C. The experimental results are summarized in Table 1. In the presence of 0.1 M KCl there is a twofold increase in the value of $k_{\text{obs}}^{\text{s}}$ and a threefold decrease in the value of $k_{\text{obs}}^{\text{f}}$ compared with the values in the absence of added salt. The reduction in $k_{\text{obs}}^{\text{f}}$ enabled us to measure activation parameters for the fast phase. The results in Table 1 also show that in the presence of KCl the two phases are of equal amplitude.

Calmodulin tryptic fragments in the presence/absence of KCl

Kinetic parameters for the Quin-2-induced dissociation of Ca^{2+} from the Ca_2 -TR₂C complex in the presence and absence of 0.1 M KCl were determined at several temperatures in the range 13.3–34.4°C. The results are summarized in Table 2. In the absence of KCl only a single process can be detected and this has an observed rate very similar to the slow phase rate observed for the whole molecule. In the presence of KCl a minor faster process is also observed. However, this process never accounts for more than 5% of the total

Table 2. Kinetic and thermodynamic parameters for the dissociation of Ca^{2+} from calmodulin fragments in the presence/absence of 0.1 M KCl at 25°C
n.d., not determined. Values in parentheses are calculated SD

Phase	Fragment	k_{obs}	Amplitude	$\Delta H^\ddagger$	$\Delta S^\ddagger$
		s^{-1}	%	$\text{kJ} \cdot \text{mol}^{-1}$	$\text{J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$
Fast	CaM ^a	650	n.d.	n.d.	n.d.
	TR ₁ C	480 (45)	> 95	28 (3.7)	-96.1 (25.0)
	CaM (+ KCl)	240 (50)	47 (3)	40.9 (5.0)	-62.7 (16.8)
	TR ₁ C (+ KCl)	280 (35)	> 95	24.3 (2.9)	-91.9 (16.8)
Slow	CaM ^a	9.1 (1.5)	n.d.	58.9 (10.5)	-29.3 (29)
	TR ₂ C	15.1 (1.2)	> 95	61.0 (4.6)	-16.7 (16.8)
	CaM (+ KCl)	24.0 (6.0)	53 (3)	63.5 (5.4)	-8.36 (20.9)
	TR ₂ C (+ KCl)	37.2 (4.3)	> 95	52.3 (5.0)	-37.6 (16.8)

^a Values from [12]; the value for k_{obs} (fast phase) of 650 s^{-1} was estimated from data at lower temperatures.

Table 3. Kinetic parameters for the dissociation of Ca^{2+} from calmodulin in the presence of trifluoperazine (13.4°C)
n.o., not observed. Values in parenthesis are the calculated SD

TFP	Phase					
	fast		intermediate		slow	
	k_{obs}	amplitude	k_{obs}	amplitude	k_{obs}	amplitude
μM	s^{-1}	%	s^{-1}	%	s^{-1}	%
0	310 (80)	47	n.o.	n.o.	3.60 (1.2)	53
15	270 (40)	32	24.2 (8)	18	0.71 (0.2)	50
	270 (50)	35	27.2 (9)	12	0.78 (0.2)	53
30	280 (60)	14	20.2 (7)	33	0.84 (0.3)	53
	280 (40)	24	14.9 (3)	30	0.67 (0.1)	46
45	300 (70)	14	15.2 (4)	34	0.66 (0.2)	52
75	n.o.	n.o.	15.6 (4)	42	0.55 (0.1)	58

amplitude and we believe that it arises from slight contamination with TR₁C. This process is more prominent in the presence of KCl because added salt slows its rate and significantly decreases the effect of amplitude losses in the instrumental deadtime.

Similar experiments were performed with TR₁C. In this case a major fast process is observed both in the presence and absence of added KCl. A minor slower process representing less than 5% of the total amplitude is observed under all conditions and we believe that it arises from minor contamination with TR₂C. The rate of the fast process is almost identical to the fast phase rate seen for the whole molecule (Table 2). Data for the minor processes is not included in Table 2.

Whole Calmodulin in the presence/absence of trifluoperazine

Kinetic parameters for the Quin-2-induced dissociation of Ca^{2+} from the Ca_4 -CaM complex in the presence of different concentrations of TFP have been measured at 13.4°C. The results are shown in Table 3 and Fig. 1. The behaviour of the slow phase process seems to be relatively simple. The addition of 15 μM TFP (TFP/CaM = 2) reduces the rate from 3.6 ± 1.2 to $0.7 \pm 0.2 \text{ s}^{-1}$; further additions of TFP cause no further reduction in the slow phase rate. Moreover, the amplitude of the slow phase rate is quite unaffected by the addition of TFP, accounting for some 50% of the total observed amplitude under all conditions.

The behaviour of the fast phase is rather more complex. The addition of TFP (15 μM) substantially reduces the

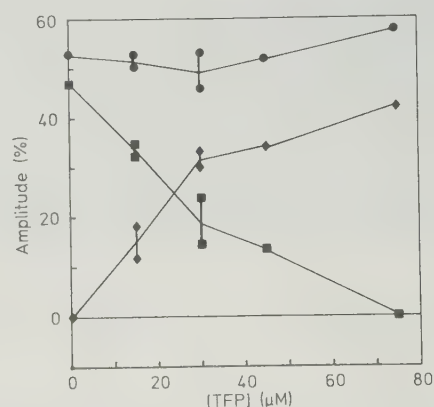


Fig. 1. Reaction amplitudes for the Quin-2-induced dissociation of Ca^{2+} from Ca_4 -CaM in the presence of added TFP. The Ca_4 -CaM complex, with varying amounts of added TFP, was mixed with Quin 2 under stopped-flow conditions to yield the following concentrations after mixing: CaM 7.5 μM , Ca^{2+} 62.5 μM and Quin 2 100 μM . The TFP concentration after mixing is plotted on the abscissa. Percentage amplitudes were calculated from the measured amplitudes following correction for dead-time losses as described in the text. (●) Slow phase; (◆) intermediate phase; (■) fast phase

amplitude of the fast phase without markedly affecting the rate. The overall reaction amplitude is not affected by the addition of TFP, the amplitude lost from the fast phase appears in a new intermediate process with a rate constant

Table 4. Kinetic parameters for the dissociation of Ca^{2+} from calmodulin in the presence of trifluoperazine and 0.1 M KCl (13.4°C)
n.o., not observed. Values in parenthesis are the calculated SD

TFP	Phase					
	fast		intermediate		slow	
	k_{obs}	amplitude	k_{obs}	amplitude	k_{obs}	amplitude
μM	s^{-1}	%	s^{-1}	%	s^{-1}	%
0	128 (28)	49	n.o.	n.o.	7.10 (1.8)	51
15	128 (18)	46	n.o.	n.o.	1.08 (0.5)	54
30	147 (24)	35	11.5 (2)	18	0.70 (0.1)	47
45	150 (40)	24	7.1 (2)	34	0.51 (0.1)	42
75	164 (35)	17	8.3 (2)	38	0.55 (0.1)	45

Table 5. Kinetic parameters for the dissociation of Ca^{2+} from fragment 1 in the presence of trifluoperazine in the presence/absence of 0.1 M KCl (25.0°C)
n.o., not observed. Values in parenthesis are the calculated SD

TFP	Phase			
	fast		intermediate	
	k_{obs}	amplitude	k_{obs}	amplitude
μM	s^{-1}	%	s^{-1}	%
0	480 (45)	>95	n.o.	n.o.
15	450 (50)	87	41.9 (8)	13
57	470 (90)	30	54.5 (9)	70
0 (+ KCl)	280 (35)	>95	n.o.	n.o.
15 (+ KCl)	275 (40)	>95	n.o.	n.o.
57 (+ KCl)	220 (15)	71	50.9 (9)	29

approximately ten times slower than the fast phase. Further additions of TFP reduce the fast phase amplitude still further with a corresponding increase in the intermediate phase amplitude. At the highest TFP concentration used (75 μM , TFP/CaM = 10) the fast phase disappears entirely and the intermediate phase accounts for one half of the total reaction amplitude (see Fig. 1). The rate of the intermediate phase is slightly decreased at high TFP concentrations, whilst the rate of the fast phase remains essentially unchanged.

Whole calmodulin in the presence of KCl, in the presence/absence of trifluoperazine

The experiments described in the previous section were repeated in the presence of 0.1 M KCl at 13.4°C; the results are qualitatively very similar to those obtained in the absence of salt. The addition of 15 μM TFP (TFP/CaM = 2) reduces the rate of the slow phase from 7.1 ± 2 to $1.1 \pm 0.5 \text{ s}^{-1}$; further additions of TFP cause no further reduction in the rate of the slow phase and the amplitude remains constant at about 50% of the total reaction amplitude. In the presence of added KCl the intermediate phase is not seen (or is too small to be accurately detected) until TFP/CaM = 4, (30 μM TFP). Again, the overall reaction amplitude does not change and losses of amplitude of the fast phase are compensated for by increases in amplitude of the intermediate phase. The conversion of fast to intermediate is not fully completed at the highest concentration of TFP used (75 μM , TFP/CaM = 10). The rate of the intermediate phase is slightly decreased

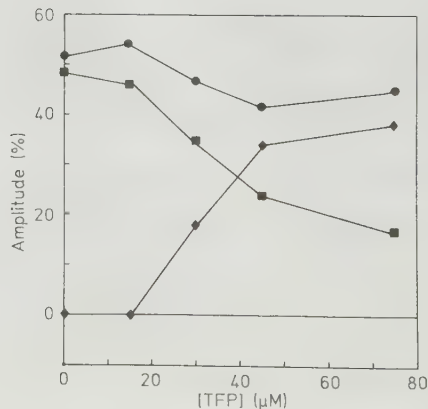


Fig. 2. Reaction amplitudes for the Quin-2-induced dissociation of Ca^{2+} from Ca_4 -CaM in the presence of added TFP and 0.1 M KCl. The Ca_4 -CaM complex, with varying amounts of added TFP, was mixed with Quin 2 under stopped-flow conditions to yield the following concentrations after mixing: CaM 7.5 μM , Ca^{2+} 62.5 μM and Quin 2 100 μM . The TFP concentration after mixing is plotted on the abscissa. Percentage amplitudes were calculated from the measured amplitudes following correction for dead-time losses as described in the text. (●) Slow phase; (◆) intermediate phase; (■) fast phase

at high TFP concentrations whilst the rate of the fast phase appears to be slightly increased. These results are summarized in Table 4 and Fig. 2.

Fig. 3 illustrates the introduction of a third kinetic phase in the presence of TFP for experiments performed in 0.1 M

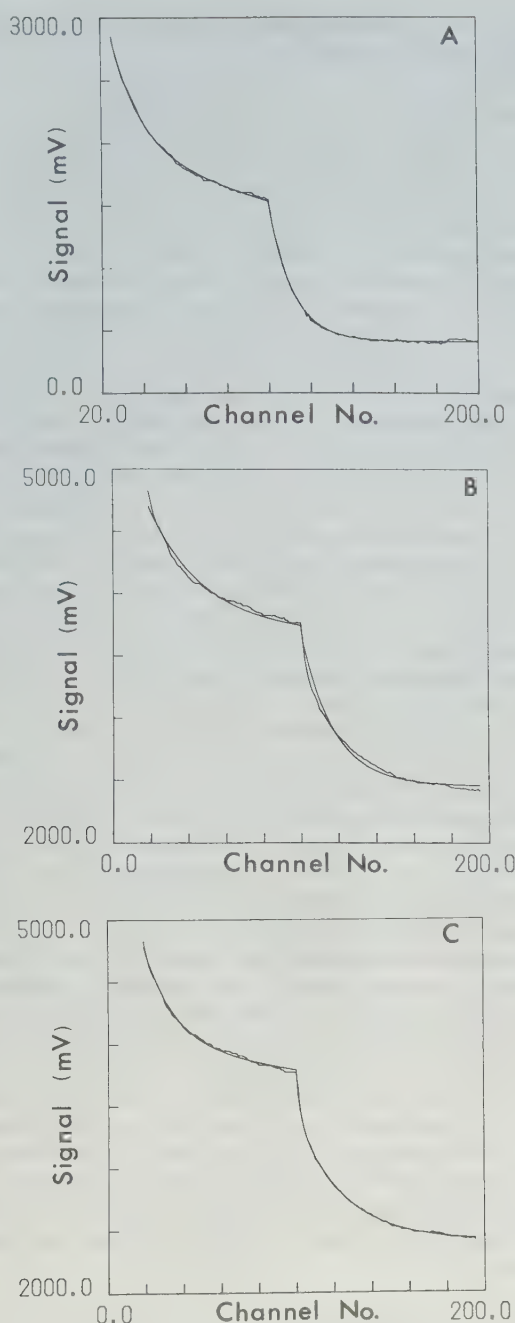


Fig. 3. Data analysis for the Quin-2-induced dissociation of Ca^{2+} from $\text{Ca}_4\text{-CaM}$ in the presence of 0.1 M KCl, with and without added TFP. Concentrations after mixing were CaM 7.5 μM , Ca^{2+} 62.5 μM and Quin 2 100 μM . (A) No added TFP. Channels 0–100, 0.1 s; 101–200, 2 s. Two-exponential fit: $k_{\text{obs}}^f = 104 \text{ s}^{-1}$, $A^f = 1170 \text{ mV}$; $k_{\text{obs}}^s = 7.9 \text{ s}^{-1}$, $A^s = 1230 \text{ mV}$; reduced $\chi^2 = 1.32$. (B) 30 μM TFP. Channels 0–100, 0.1 s; 101–200, 10 s. Two-exponential fit: $k_{\text{obs}}^f = 71.7 \text{ s}^{-1}$, $A^f = 1050 \text{ mV}$; $k_{\text{obs}}^s = 1.16 \text{ s}^{-1}$, $A^s = 1250 \text{ mV}$; reduced $\chi^2 = 9.06$. (C) Same data as B, three-exponential fit: $k_{\text{obs}}^f = 125.0 \text{ s}^{-1}$, $A^f = 710 \text{ mV}$; $k_{\text{obs}}^m = 10.3 \text{ s}^{-1}$, $A^m = 580 \text{ mV}$; $k_{\text{obs}}^s = 0.81 \text{ s}^{-1}$, $A^s = 1110 \text{ mV}$; reduced $\chi^2 = 1.18$.

KCl. Fig. 3A shows the two-exponential fit for the experiment in the absence of TFP (reduced $\chi^2 = 1.32$). In the presence of TFP (30 μM) a two-exponential fit is no longer adequate (Fig. 3B, reduced $\chi^2 = 9.08$) whereas the three-exponential fit represents this experimental data very well (Fig. 3C, reduced $\chi^2 = 1.18$). The ability to resolve the three exponentials

is due to the separation of the observed rates by at least one order of magnitude.

Calmodulin tryptic fragment 1 in the presence/absence of KCl and trifluoperazine

Kinetic parameters for the Quin-2-induced dissociation of Ca^{2+} from the $\text{Ca}_2\text{-TR}_1\text{C}$ complex in the presence of different concentrations of TFP (in the presence and absence of 0.1 M KCl) have been measured at 25°C. The results are summarized in Table 5. The results are similar to those observed with the whole molecule except for the simplification that arises from the absence of the slow phase. The addition of increasing amounts of TFP leads to the loss of fast phase amplitude with the parallel appearance of a slower phase which is evidently comparable to the intermediate phase seen for the whole molecule.

As was the case with the whole molecule the effects of TFP seem to be somewhat more marked in the absence of KCl, e.g. in the presence of 57 μM TFP (TFP/CaM = 8) the intermediate phase accounts for 70% of the total amplitude in the absence of KCl compared with only 29% in the presence of the salt.

DISCUSSION

Ca^{2+} dissociation from whole calmodulin

In a previous communication [12] we reported that the dissociation of Ca^{2+} from calcium-saturated calmodulin ($\text{Ca}_4\text{-CaM}$) takes place in two distinct steps. This result, obtained at low temperatures by the stopped-flow method used here, is confirmed and extended in the present work. Under conditions of low ionic strength the rates of the slow and fast dissociative processes differ by two orders of magnitude (cf. Table 1). The present study shows that the dissociation of the $\text{Ca}_4\text{-CaM}$ complex is qualitatively similar at higher ionic strengths, although the rate constants are affected. The effect of 0.1 M KCl is to accelerate the slow process by a factor of about two while the fast process is slowed by a factor of about three.

The demonstration that each of the two kinetic processes accounts for 50% of the total reaction amplitude, in the absence as well as in the presence of 0.1 M KCl, convincingly shows that they each correspond to the release of two Ca^{2+} ions. It seems reasonable to attribute the two distinct rate processes to the dissociation of Ca^{2+} from two strong and two weak bindings sites respectively. This interpretation is in line with our stopped-flow results on the proteolytic fragments of CaM to be discussed below. A subdivision of the four Ca^{2+} binding sites of CaM into two strong and two weak sites has also been inferred from ^1H , ^{43}Ca and ^{113}Cd NMR studies at low ionic strength [14, 16, 20–25]. The present study indicates that increased ionic strength does not qualitatively change this general picture of the Ca^{2+} -binding properties of CaM, contrary to what has been suggested recently [11].

To what extent the changes in the Ca^{2+} dissociation rates caused by 0.1 M KCl reflect changes in the binding constants depends on the magnitude of the changes in the association rate constants. These rates are too fast to be measured directly [26] but may be estimated, from the appropriate binding constants and dissociation rate constants, to be of the order of $10^7\text{--}10^8 \text{ M}^{-1} \text{ s}^{-1}$ [27]. According to the Eigen-Tamm mechanism, the rate of association of a small ligand with a cation may be written as the product of an outer sphere

association constant for the reaction partners, K_{os} , and a first-order rate constant characterizing the dissociation of a water molecule from the inner hydration sphere of the cation, k_{is} [28]. For Ca^{2+} the value of k_{is} is generally taken to be $3 \times 10^8 \text{ s}^{-1}$. CaM is a highly acidic protein and even with four Ca^{2+} ions bound will carry a large negative charge ($z \approx -15$) at neutral pH. Since the tertiary structure of CaM is not known the effect of the spatial distribution of these charges can only be approximated. It appears likely, however, that the attractive interaction between Ca^{2+} ions and negatively charged amino acid side chains of the Ca^{2+} -binding loops of the protein will result in values of K_{os} exceeding unity. Thus the rate of Ca^{2+} association inferred from experiment is lower than that predicted from the Eigen-Tamm scheme, implying that the dissociation of a water molecule from the inner hydration sphere of Ca^{2+} may not be the rate-limiting step. Therefore, local structural changes in the Ca^{2+} binding region of the protein may be the rate-limiting step. If so, increased ionic strength of the medium, which would normally lead to a reduction in the association rate constant by decreasing the value of K_{os} , may be relatively ineffective. The results reported here could therefore imply that the affinity of the strong sites for Ca^{2+} is decreased by the presence of KCl whilst that of the weak sites is increased. In fact, Haiech et al. [29] inferred that added KCl leads to a decrease in the affinity of Ca^{2+} for all sites. However, it must be noted that no direct experimental information about the changes in association rate constants induced by adding 0.1 M KCl is currently available.

In Table 1 we compare the activation parameters obtained by NMR and stopped-flow measurements. The parameters determined by the two methods for the slow dissociation process in the absence of KCl have previously been shown to be in good agreement [12]. In the presence of 0.1 M KCl it was possible, in the present study, to measure activation parameters for both the slow and fast processes. For the fast process the stopped-flow values of $\Delta H^\ddagger (41 \pm 5 \text{ kJ} \cdot \text{mol}^{-1})$ and $\Delta S^\ddagger (-63 \pm 17 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1})$ are in good agreement with values determined by ^{43}Ca NMR [19].

The value of $\Delta S^\ddagger$, the entropy of activation, may be compared with the overall entropy change for the binding of Ca^{2+} ions as determined from thermochemical data [10]. The average entropy change per mol of Ca^{2+} bound to CaM, $\Delta S_{\text{CaM}}^\circ$, is ca. $66 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$. This value is virtually identical to that obtained for the binding of Ca^{2+} to the fragment TR_1C ; $\Delta S_{\text{TR}_1\text{C}}^\circ = 60 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$. Remembering that the reaction observed in this work is the reverse of that observed in the thermochemical studies we note the striking fact that the entropy of activation closely agrees with the overall entropy change for the reaction. This suggests that the transition state for the dissociation reaction has properties similar to those of the initial stages of Ca^{2+} binding, i.e. a Ca^{2+} ion that is highly hydrated and the protein in a state close to that of the Ca^{2+} -free form.

In this respect the Ca^{2+} complexes with CaM show a striking similarity to Ca^{2+} complexes of small organic multidentate ligands involving carboxylic acid groups such as, for example, EDTA or Quin 2. From the dissociation kinetics of the Ca^{2+} -Quin 2 complex we have obtained $\Delta S^\ddagger = -126 \pm 10 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$. A similar value ($-142 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$) was obtained by Quast et al. [30]; these authors also obtained a value of $134 \pm 50 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ for ΔS° , the overall entropy change. Although the latter value is not very accurate it is similar to values obtained for related ligands (e.g. 130, 96 and 100 for EDTA, EGTA and EEDTA, respectively [31]). The agreement between the value for the

overall entropy change and that for the entropy of activation is nonetheless striking.

Ca^{2+} dissociation from the tryptic fragments, TR_1C and TR_2C : strong and weak cation binding sites

Tryptic cleavage of CaM in the presence of Ca^{2+} gives two fragments of equal size [16, 32–34], TR_1C (1–77) encompassing Ca^{2+} -binding domains I and II and TR_2C (78–148) encompassing Ca^{2+} -binding domains III and IV. In order that stopped-flow studies of proteolytic fragments of CaM be a valid approach for assigning which of the four Ca^{2+} -binding sites of CaM are slow and which are fast a prerequisite is that the cleavage should leave the two halves of CaM unaffected. For the case of TR_1C and TR_2C the requirement seems to be fulfilled. Studies using several biophysical techniques have shown that the equilibrium properties of CaM are well represented by a summation of the properties of the fragments [16, 25, 34, 35].

As can be seen from Table 2 there is a good agreement between the data for TR_1C and that for the rapidly dissociating sites in CaM and between those for TR_2C and the corresponding slowly dissociating sites. Not only are the dissociation rates very similar but also the effect of KCl on the dissociation rates – for TR_1C the Ca^{2+} dissociation rate decreases with increasing KCl concentration, whereas for TR_2C the dissociation rate increases. The rapidly dissociating sites ($k_{\text{off}} = 650 \text{ s}^{-1}$; see Table 1) are assumed to be the weakly binding sites (with $K_a < 10^6 \text{ M}^{-1}$ [29]) since a stability constant of, say, $\geq 5 \times 10^6 \text{ M}^{-1}$ (strongly binding sites, [29]) would imply an association rate constant of $(5 \times 10^6 \times 650) \geq 3 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$, which is unreasonably large. However, it must be noted that the absolute values of the stability constants are not established unambiguously. We therefore identify domains I and II as the weakly binding (rapidly dissociating) and domains III and IV as the strongly binding (slowly dissociating) sites. This assignment is in complete agreement with results from several NMR studies [14, 16, 20, 24, 25]. It should be mentioned that results obtained in studies in which lanthanide (Ln^{3+}) ions are used as calcium probes have been interpreted in a completely different way [36–39]. We strongly believe this interpretation to be invalid, however, following the demonstration that Ln^{3+} ions do not bind in the same order as does Ca^{2+} [40] (W. Niemczura, unpublished results).

Effects of trifluoperazine on the Ca^{2+} dissociation rates

The time dependence of the Quin 2 fluorescence intensity changes observed during the dissociation of Ca^{2+} from $\text{Ca}_4\text{-CaM}$ in the presence of TFP is best represented as a sum of three exponentials. The slowest phase, which has an amplitude corresponding to two Ca^{2+} ions under all experimental conditions (with added salt or TFP), is attributed to dissociation from the strong sites (III and IV). The effect of added TFP is to decrease this rate by a factor of 5 at all TFP concentrations used in the present study. The lowest TFP/CaM ratio was 2, which makes it possible to estimate a lower limit for the TFP binding constant to CaM of $K > 10^6 \text{ M}^{-1}$. It seems plausible that the first TFP molecule binds to the C-terminal half of CaM.

The sum of the amplitudes for the two faster phases in the stopped-flow experiments always corresponds to two Ca^{2+} ions and both are therefore attributed to the weak sites (I and II). If we assume that the intermediate phase, which is only

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Paper IV

Kinetics of Ca^{2+} Binding to Calmodulin and Its Tryptic Fragments Studied by ^{43}Ca NMR

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Key words: Calmodulin; Tryptic fragments; ^{43}Ca NMR; Ca^{2+} binding;
 Ca^{2+} exchange rates; (Kinetics)

Proofs and correspondence to Anita Teleman

Abbreviations:

CaM	calmodulin
NMR	nuclear magnetic resonance
TR ₁ C	fragment 1-77
TR ₂ C	fragment 78-148

Summary

The kinetics of calcium ion binding to bovine testis calmodulin and its tryptic fragments have been studied by ^{43}Ca NMR. The same subdivision of the Ca^{2+} binding sites of calmodulin into two with slow exchange (high affinity) and two with fast exchange (low affinity) observed at low ionic strength is also encountered at high ionic strength. The effect of 0.1 M KCl is to reduce the exchange rate of the fast process from 1150 s^{-1} to 520 s^{-1} at $25\text{ }^{\circ}\text{C}$.

Studies of the tryptic fragments TR_1C and TR_2C , comprising the N- or C-terminal half of calmodulin, respectively, clearly identified Ca^{2+} binding domains I and II as those with fast exchange (low affinity) and domains III and IV as those with slow exchange (high affinity).

Activation parameters are reported for calmodulin and TR_1C .

Correlation times have been measured for calcium ions bound to the fragments. The obtained values, 5 and 6 ns for TR_1C and TR_2C , respectively, are of the same order as rotational correlation times for the entire fragment molecules, indicating that the calcium ions do not have any mobility with a correlation time in the ns range within the sites.

Introduction

Calcium is a major regulator of a wide variety of cellular events [1,2]. In many instances the regulatory function appears to be mediated by the binding of calcium ions to the small ($M_r = 16,700$) acidic protein calmodulin (CaM) [3]. The binding process is accompanied by a conformational change which induces CaM to bind to and alter the biological activity of a number of enzymes, ion pumps, receptors and other proteins (for a review see [4]). The rate by which these cellular events is modulated will depend on many factors including the diffusion, binding and release of calcium ions to and from calmodulin. Quantitative information on these dynamic processes is therefore important for our understanding of the biological activity of calcium ions. In the present work we have studied this problem using ^{43}Ca NMR as a tool.

The three-dimensional structure of CaM has recently been reported by Babu et al. [5]. In the crystal, the molecule consists of two globular lobes connected by an α -helix. The two lobes are of equal size and each contains two calcium binding sites. The arrangement of a calcium binding site is found to be helix-calcium binding loop-helix, i.e. an "EF-hand" structure [6], as observed also in parvalbumin [6], intestinal calcium binding protein [7] and troponin-C [8,9].

The binding of Ca^{2+} ions to the four sites (numbered I-IV from the N-terminal) in CaM has been studied by several groups. Different techniques have been employed to obtain information about binding constants, such as equilibrium and/or flow dialysis, and changes induced in the protein structure upon Ca^{2+} binding, e.g. NMR, UV absorption, circular dichroism and fluorescence (for a recent review see [10]). By contrast, only limited information is available on the rates of association and dissociation of Ca^{2+} ions in Ca^{2+} -CaM complexes. Direct information on the kinetics of calcium ion binding to CaM can be obtained by ^{43}Ca NMR. We have earlier shown that a ^{43}Ca NMR signal from calcium ions bound to CaM can be observed [11] and that the temperature dependence of the NMR signal from calcium saturated CaM is typical for intermediate chemical exchange, which permitted rate constants to be determined with fairly high accuracy [12,13]. In the present work these kinetic studies are considerably extended so that not only native CaM but also the tryptic fragments TR_1C and TR_2C , that correspond closely to the N-terminal and C-terminal half of CaM, respectively, have been studied. The kinetic data for the fragments enables the assignment of the two different rate processes observed for CaM to different pairs of Ca^{2+} -binding sites. Also effects of ionic strength on the ^{43}Ca NMR signals and on the calcium exchange rates have been investigated. Finally, we have also measured the rotational correlation times of the tryptic fragments.

Experimental Procedures

Materials

Bovine testis calmodulin (CaM) and its two tryptic fragments TR₁C and TR₂C were prepared as previously described [14,15]. Isotopically enriched ⁴³Ca (CaO enriched to 60% in ⁴³Ca) was purchased from Technabexport, Moscow, USSR through their Swedish sales agent Matreco AB, Södertälje, Sweden. All chemicals used were of the highest quality commercially available.

Methods

The purity of CaM and its proteolytic fragments was checked using both sodium dodecyl sulfate and agarose gel electrophoresis in the presence of excess EDTA or Ca²⁺. Ca²⁺-free proteins were prepared by passing the protein solution through a Chelex-100 column; the residual calcium content was determined by atomic absorption spectroscopy (≤ 0.05 Ca²⁺/protein). The absence of EDTA in the protein solutions was checked by ¹H NMR at 360 MHz.

The ⁴³Ca NMR spectra were recorded at 24.35 MHz on a Nicolet 360 WB NMR spectrometer. The probe used [13] was designed and built in our laboratory and possesses a solenoid coil. A pulse sequence, designed to decrease the effect from probe ringing, was used for some spectra [16]. The 90° pulse length was normally 30 μ s, the preacquisition delay was 50 μ s and the acquisition time was varied between 25.6 and 204.8 ms. Up to 2.2×10^6 transients were accumulated in a total time of 17 hours. The longitudinal relaxation time, T₁, was determined by the inversion-recovery technique [17] and the transverse relaxation time, T₂, was obtained from the line width at half-height, $\Delta\nu_{1/2}$, from the relation $T_2 = 1/(\pi \cdot \nu_{1/2})$.

Data treatment

The primary NMR data obtained at different temperatures were treated using total bandshape analysis [13,16]. The quadrupole coupling constant, χ_e , was calculated from the observed line width using an equation derived by Halle and Wennerström [18]

$$\frac{1}{T_2} = \frac{3\pi^2}{100} \chi_e^2 \frac{2I+3}{I^2(2I-1)} \left[3\tau_c + \frac{5\tau_c}{1+(\omega_0\tau_c)^2} + \frac{2\tau_c}{1+4(\omega_0\tau_c)^2} \right] \quad (1)$$

where I is the spin, ω_0 the Larmor frequency and τ_c the correlation time. This equation applies when $\omega_0\tau_c < 1.5$ [11,18]. In our study the longest correlation time encountered is that for CaM, estimated to be 8.2 ns at 23 °C [11]. This gives $\omega_0\tau_c = 1.2$ in the Ca^{2+} -titration performed at room temperature (24 °C), whereas $\omega_0\tau_c$ will become slightly larger than 1.5 at the lowest temperatures studied. Equation (1) can, however, with some confidence be used also at the low temperatures, since the Ca^{2+} -exchange rate is sufficiently slow that the observed resonance from the free ions is only moderately broadened by bound ions. To obtain the best least-squares fit of calculated spectra to experimental, $\Delta H^\#$, $\Delta S^\#$, χ_e and the chemical shifts were varied by means of a simple iterative procedure. The chemical shifts were allowed to vary slightly with temperature whereas T_2 for free Ca^{2+} and the fraction of bound Ca^{2+} were assumed to be temperature independent.

The correlation time was calculated from the T_2/T_1 ratio and equation (2) [18].

$$\frac{T_2}{T_1} = 2 \left[\frac{1}{1+(\omega_0\tau_c)^2} + \frac{4}{1+4(\omega_0\tau_c)^2} \right] / \left[3 + \frac{5}{1+(\omega_0\tau_c)^2} + \frac{2}{1+4(\omega_0\tau_c)^2} \right] \quad (2)$$

Results

Temperature Dependence of the ^{43}Ca NMR Signal from Free Ca^{2+}

The chemical shift of the ^{43}Ca NMR signal of free Ca^{2+} in a 40 mM $\text{Ca}(\text{ClO}_4)_2$ solution was found to be temperature dependent with a nearly linear variation from 0.54 ppm at 5 °C to -0.67 ppm at 65 °C. The chemical shift at room temperature was defined as 0 ppm. In the simulations of the temperature dependences below, we have used a variation of 0.5 Hz/°C for the chemical shift of the resonance from free Ca^{2+} .

Calmodulin

The line width of the ^{43}Ca NMR signal of CaM in 0.1 M KClO_4 as a function of the Ca^{2+} to CaM ratio is presented in Fig. 1A. Below two equivalents of Ca^{2+} per CaM a 650 Hz broad resonance is observed at 7.5 ppm downfield to the resonance of free Ca^{2+} . Using a correlation

time of 8.2 ns [11] the quadrupole coupling constant, χ_e , for the bound ions can be calculated to 1.1 MHz.

Between 2 and 4 mol Ca^{2+} added per mol CaM, the line width of the resonance remains 650 Hz, whereas the chemical shift gradually changes towards the value of free Ca^{2+} as the ratio of Ca^{2+} to CaM increases.

Above 4 mol Ca^{2+} per mol CaM, the observed resonance becomes non-Lorentzian. The resonance seems to be composed of a 650 Hz broad component and a narrower component. The relative intensity of the broad component decreases whereas that of the narrow one increases as the ratio of Ca^{2+} to CaM is increased. The narrower resonance represents free Ca^{2+} and is broadened due to exchange with protein-bound Ca^{2+} . The line width presented in Fig. 1A is obtained from a Lorentzian fit to the total resonance.

The temperature dependence of CaM in 0.1 M KClO_4 is shown in Fig. 1B. In the temperature range 3 - 70 °C the line width variation is typical of a situation where we go from slow via intermediate to fast exchange with increasing temperature. The temperature dependence was simulated according to three models: (i) two calcium ions exchanging whereas the other two are assumed to exchange too slowly to influence the line width in the temperature range studied (ii) two calcium ions exchanging with intermediate rates and two exchanging slowly but still sufficiently fast to affect the bandshape at the higher temperatures (iii) all four calcium ions exchanging with the same rate. The first model gives a reasonable fit to the experimental data over the whole temperature range, but with the quadrupole coupling constant obtained, the line width of the protein-bound calcium ions is calculated to be about 10% broader than observed at low Ca^{2+} to CaM ratios. The second model results in the best overall agreement with the experimental data as can be seen from Fig. 1. The kinetic parameters are $\Delta H^\#_f = 39$ kJ/mol, $\Delta H^\#_s = 49$ kJ/mol and $\Delta S^\#_{f,s} = -63$ J/mol·K. These values correspond to $k^\#_{\text{off}} = 520$ s⁻¹ and $k^\#_{\text{off}} \approx 10$ s⁻¹ at 25 °C for the faster and slower exchange rates, respectively. The rate constant for the rapidly exchanging Ca^{2+} is well defined, whereas the rate constant for the slowly exchanging Ca^{2+} can be varied between 5 and 25 s⁻¹ without significantly affecting the overall fit. The third model resulted in a poorer fit and the calculated quadrupole coupling constant was much lower than that obtained at low Ca^{2+} concentrations.

The N-terminal Tryptic Fragment of Calmodulin (TR₁C)

At low ionic strength one 570 Hz broad resonance at ≈ 2 ppm is observed in the ^{43}Ca NMR spectra at subsaturating Ca^{2+} levels. Above two equivalents of Ca^{2+} per TR₁C the resonance,

which appears to be Lorentzian, is displaced towards the shift of free Ca^{2+} and the line width decreases due to chemical exchange with free Ca^{2+} , cf Fig. 2A.

The temperature dependence of the ^{43}Ca NMR line width at low ionic strength is shown in Fig. 2B. The Ca^{2+} exchange is intermediately fast at room temperature. A calculation using a model with two Ca^{2+} ions per protein exchanging with the same rate and a quadrupole coupling constant of 1.1 MHz, gives a good fit between 3 and 35 °C. The calculated curve fits poorly above 40 °C, i.e. the calculated line widths are less than the experimental values. If the quadrupole coupling constant is allowed to vary the same rate parameters are obtained, whereas the quadrupole coupling constant obtained is much too high to agree with experimental data at low Ca^{2+} to CaM ratios. The resulting "best fit" of Ca^{2+} exchange rate parameters are, at 25 °C: $\Delta H^\ddagger = 39 \text{ kJ/mol}$, $\Delta S^\ddagger = -63 \text{ J/mol}\cdot\text{K}$ and $k_{\text{off}} = 430 \text{ s}^{-1}$.

In the presence of 0.1 M KCl a titration of TR_1C with ^{43}Ca gives rise to one 560 Hz broad ^{43}Ca NMR signal at $\approx 5 \text{ ppm}$ up to two equivalents of Ca^{2+} per TR_1C . At higher Ca^{2+} to TR_1C ratios the resonance, which appears to be Lorentzian, shifts towards the value of free Ca^{2+} and the line width decreases due to chemical exchange with free Ca^{2+} , see Fig. 3A.

Fig. 3B shows the temperature dependence of the ^{43}Ca NMR line width in the presence of 0.1 M KCl. The temperature dependence can be well simulated in the temperature range 3-45 °C assuming two identically exchanging Ca^{2+} ions per protein and a quadrupole coupling constant of 1.1 MHz. A poor fit is obtained above 45 °C, the line widths of the experimental resonances being larger than the calculated ones. As in the case of low ionic strength, a too high value of the quadrupole coupling constant is obtained when it is allowed to vary in the fitting of the temperature dependence, but the calculated exchange rate is essentially unaffected. The resulting Ca^{2+} exchange rate parameters are, at 25 °C: $\Delta H^\ddagger = 33 \text{ kJ/mol}$, $\Delta S^\ddagger = -84 \text{ J/mol}\cdot\text{K}$ and $k_{\text{off}} = 470 \text{ s}^{-1}$.

From an inversion recovery experiment on a sample containing 0.55 mM TR_1C and 0.84 mM Ca^{2+} the longitudinal relaxation time, T_1 , was calculated to be 1.1 ms. No deviation from single exponential behaviour was detected. The line width at half height (560 Hz) corresponds to an effective transverse relaxation time $T_2 = 0.57 \text{ ms}$. All the data were measured at 26 °C. Using these relaxation data, we calculate a correlation time of 5 ns and a quadrupole coupling constant of 1.1 MHz from equations (1) and (2).

The C-terminal Tryptic Fragment of Calmodulin (TR_2C)

Upon titration of TR_2C with $^{43}\text{Ca}^{2+}$ a 520 Hz broad resonance at a chemical shift of $\approx 9 \text{ ppm}$ downfield relative to the signal of free Ca^{2+} is observed in the ^{43}Ca NMR spectra up to a

ratio of almost two equivalents of Ca^{2+} per TR_2C , part A of Fig. 4. The line shape appears to be Lorentzian. Further addition of Ca^{2+} results in a spectrum of two overlapping resonances, indicative of slow exchange between "free" and strongly bound Ca^{2+} . The narrow resonance is about 75 Hz wide at room temperature (22 °C).

An increase of the temperature from 22 °C to 50 °C results in no observable changes in the line widths, the chemical shifts or the relative intensities of the two resonances. A lowering of pH from 7.4 to 5.0 does not change the relative intensities of the two resonances. A precipitate was formed as the pH was lowered to 4.5.

Fig. 5A shows some partially relaxed ^{43}Ca NMR spectra of the protein-bound Ca^{2+} . In Fig. 5B the intensity of the resonance is plotted as a function of the delay time, τ , and the result of fitting the data to a single exponential is shown as the solid curve corresponding to the longitudinal relaxation time $T_1 = 1.3$ ms. The effective transverse relaxation time was estimated from the ^{43}Ca NMR signal line width to be $T_2 = 0.61$ ms. All data were measured at 26°C. From these data and equations (1) and (2), a correlation time of 6 ns and a quadrupole coupling constant of 1.1 MHz were calculated.

Discussion

TR_1C and TR_2C : Assignment of the Fast and Slow Calcium Exchange Rates

The chemical shift range in ^{43}Ca NMR hitherto observed for Ca^{2+} ions bound to proteins is only about 20 ppm [20]. Due to this fact and the observation that resonances from this quadrupolar ion bound to proteins are broad, 200-1000 Hz, it has rarely been possible to obtain resolved resonances from Ca^{2+} ions bound to different sites in a protein [21]. In the case of CaM only one resonance is observed at subsaturating Ca^{2+} levels. However, a number of observations indicate the existence of two kinds of Ca^{2+} sites in CaM with different kinetic behaviour. Firstly, the chemical shift changes towards the value of free Ca^{2+} on going from two to four equivalents of Ca^{2+} added per CaM, which we interpret as being caused by a chemical shift difference between two different pairs of Ca^{2+} binding sites. In line with this, the resonances from calcium ions bound to TR_1C and TR_2C are observed at different chemical shifts, see Table 1. Secondly, the best overall agreement with experimental data for the temperature dependence of the ^{43}Ca NMR resonance of calcium saturated CaM is obtained with a model of two pairs of Ca^{2+} binding sites, where the pairs have widely different Ca^{2+} kinetics. Thirdly, it is possible to detect two overlapping resonances above four equivalents of Ca^{2+} per CaM, one broad from Ca^{2+} ions

bound to high-affinity binding sites in CaM (slow exchange) and one rather narrow resonance due to Ca^{2+} ions undergoing exchange between low-affinity binding sites in CaM (fast exchange) and "free". (The term "free" has only a relative meaning. Ca^{2+} ions will interact weakly with carbonyl and/or carboxylate oxygen atoms on the surface of the protein.)

In order to assign the fast and slow exchange processes to the two halves of CaM, we may use the kinetic properties of the proteolytic fragments TR_1C and TR_2C . The tryptic fragmentation of Ca^{2+} saturated CaM takes place mainly at Lys-77 [22], which is in the middle of the long exposed α -helix that connects the two globular lobes [5]. In the crystal structure no contact other than the α -helix is observed between the two lobes, suggesting that the cleavage should leave the two halves of CaM unaffected. In fact a number of studies using several biophysical techniques have shown that the spectroscopic and other properties of CaM are well represented as a sum of the properties of the fragments [22-26]. These studies also indicate that the structure of CaM in solution is similar to that elucidated by X-ray crystallography. However, the two fragments can not mimic the modulating capabilities of calmodulin [27-30].

There is good agreement between the kinetic data for TR_1C and the fast rate process observed for CaM on the one hand and between those for TR_2C and the slow process in CaM on the other, as can be seen from Tables 1 and 2. We therefore identify Ca^{2+} binding domains I and II as the Ca^{2+} binding sites with fast exchange (low affinity) and domains III and IV as the Ca^{2+} binding sites with slow exchange (high affinity). This assignment is consistent with results from several other studies using NMR as well as stopped-flow techniques [14,22,24,26,32,33]. In line with these solution studies, lead ions will substitute for calcium ions only in domains I and II in the CaM crystal [5].

As can be seen from Fig. 2 and 3 it is not possible to obtain a good fit for TR_1C between experimental and calculated points over the whole temperature interval with the assumption of a temperature independent coupling constant of 1.1 MHz, which is the value obtained at less than saturating levels of Ca^{2+} ions. A possible explanation for this is that a conformational change of TR_1C occurs at elevated temperatures which results in an increase in the quadrupole coupling constant for the protein-bound Ca^{2+} ions. A 10% increase in the quadrupole coupling constant in the high temperature conformation compared to the low temperature conformation is sufficient to explain the deviation between experimental and calculated line widths. Fortunately, the exchange rate is determined from the data at lower temperatures, leading to well defined calculated exchange rates.

The slow exchange behaviour observed for TR_2C is consistent with earlier results obtained

with ^1H and ^{113}Cd NMR [14,22]. The additional ^{43}Ca NMR signal observed above saturating Ca^{2+} ion concentrations is broad (line width 75 Hz) compared with the resonance of Ca^{2+} in the absence of protein (line width 5 Hz under identical experimental conditions). This broadening is presumably due to interaction of Ca^{2+} with carbonyl and/or carboxylate oxygen atoms on the surface of the protein. From the temperature independence of the ^{43}Ca NMR line width, we estimate the off-rate of Ca^{2+} ions from the two TR_2C sites to be less than 100 s^{-1} at $50\text{ }^\circ\text{C}$.

In the temperature dependence of the ^{43}Ca NMR resonance of calcium ion saturated CaM, the best overall agreement with experimental data was achieved when the two slowly exchanging calcium ions exchanged fast enough to affect the bandshape at higher temperatures. However, another interpretation of the broader experimental line widths at higher temperatures is plausible, in view of the results from the tryptic fragments. A conformational change of the N-terminal half of CaM, similar to the conformational change proposed for TR_1C , may occur at elevated temperatures. The slow exchange rate for CaM will then be reduced.

The correlation times, 5 and 6 ns for TR_1C and TR_2C , respectively, are of the same order as those calculated from the Debye-Stokes-Einstein relation using estimated radii of the fragments from the crystal structure of CaM. Therefore we conclude that the calcium ions do not have any mobility with a correlation time in the ns range within the sites.

Effects of Ionic Strength

In previous publications [12,13] we have reported measurements of the exchange rate of Ca^{2+} from calmodulin at low ionic strength. The two observed exchange rates were determined to $k_{\text{off}}^f = 1.0 \times 10^3\text{ s}^{-1}$ and $k_{\text{off}}^s \approx 30\text{ s}^{-1}$ at $23\text{ }^\circ\text{C}$. The superscripts f and s refer to the faster and slower exchange rates, respectively. Here we have studied the effect of high ionic strength on the exchange rates as measured by ^{43}Ca NMR. Earlier, we have shown that with ^{113}Cd NMR, qualitatively the same results are obtained when a Cd^{2+} titration to CaM is performed at low or high ionic strength [34,35].

The NMR data strongly indicate that we are faced with the same subdivision of the Ca^{2+} sites of CaM into two sites with high-affinity and two with low-affinity at high as well as at low ionic strength. The exchange rate of the fast process is slowed down by a factor of two for CaM in the presence of 0.1 M KClO_4 , Table 2. The same effect of ionic strength has been observed in a recent stopped-flow measurement in the presence of 0.1 M KCl [26]. No definite effect on the slow dissociation rate can be observed, mainly since this rate is too slow to be well defined from the NMR experiment. No effect of 0.10 M KCl on the exchange rate is observed in the case of TR_1C .

From the observations above we conclude that the Ca^{2+} exchange rates and the order in

which the sites are filled by Ca^{2+} do not appear to be very sensitive to ionic strength. This is contrary to what has been suggested by Burger et al [36], who obtained a reasonably good fit to their data, monitored at high ionic strength, with a model of four identical, independent sites. However, a careful simulation of Ca^{2+} binding to CaM [37] shows that an excellent fit to the experimental data can equally well be obtained with two classes of Ca^{2+} binding sites, provided the binding constants are properly chosen and with the assumption of a certain degree of positive cooperativity between the two sites in each class. Such cooperativity is in fact strongly indicated by NMR studies [14,38] as well as other biophysical techniques [10]. With the model of two classes of sites, all experimental data on calcium ion binding to CaM reported to date can be accounted for.

Comparison of Kinetic Data Obtained with ^{43}Ca NMR and Stopped-flow Measurements

Stopped-flow studies using a fluorescence chelator for Ca^{2+} and ^{43}Ca NMR give complementary information regarding the dissociation of Ca^{2+} from CaM. ^{43}Ca NMR provides a good measure in particular of the fast exchange process. This rate is close to the limit for stopped-flow studies. On the other hand, the slow exchange process can be measured with high accuracy by the stopped-flow method. Even at elevated temperatures the slow exchange rate is so slow that it is impossible to obtain more than an approximate value with ^{43}Ca NMR.

The dissociation rates determined by stopped-flow techniques for TR_1C [26] and the fast component of CaM [26,31] are a factor of two slower than the corresponding rates determined by ^{43}Ca NMR, see Table 2 (except for TR_1C at low ionic strength where the same value is reported). Although this difference is barely significant in view of the combined error, it may be because the two techniques actually measure exchange rates under somewhat different conditions. By contrast to the stopped-flow experiment, the NMR experiment determines the exchange rate under equilibrium conditions. It may be anticipated that a Ca^{2+} ion dissociating from a site in the protein may return to the protein or another protein molecule before it is captured by the fluorescence probe. With ^{43}Ca NMR all exchanges between bound and free Ca^{2+} ions will be detected. Therefore, the dissociation rate obtained from stopped-flow experiments is a lower limit for the rate constant. The somewhat higher exchange rates obtained with NMR as compared to stopped-flow can thus be easily understood.

In Table 2 we compare the activation parameters obtained by NMR and stopped-flow measurements. The $\Delta G^\ddagger$ values are in good agreement, being about 58 kJ/mol for the fast phase and about 66 kJ/mol for the slow phase. In all cases the entropy of activation is strongly negative.

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Table 1. Some ^{43}Ca NMR data for calmodulin and its tryptic fragments. For calmodulin the shown values are for the two high affinity calcium ion binding sites. I, ionic strength.

Protein	Medium	δ (ppm)	$\Delta\nu_{1/2}$ (Hz)	χ_e (MHz)
CaM ^a	low I	+10	770	1.15
CaM	0.1 M KClO ₄	+7.5	650	1.1
TR ₁ C	low I	+2	570	1.1
TR ₁ C	0.1 M KCl	+5	560	1.1
TR ₂ C	low I	+9	520	1.1

^aValues from Andersson et al. [11].

Table 2. Kinetic and thermodynamic parameters for the dissociation of Ca^{2+} from calmodulin and its tryptic fragments. The values are determined by ^{43}Ca NMR or stopped-flow (low or high ionic strength at 25 °C). nd, not determined.

Phase	Method	Protein	k_{obs} (s^{-1})	$\Delta H^\#$ (kJ/mol)	$\Delta S^\#$ (J/mol·K)	$\Delta G^\#$ (kJ/mol)
Fast	NMR	CaM^{a}	1150	43	-42	56
	SF	CaM^{b}	650	nd	nd	nd
	NMR(0.1 M KClO_4)	CaM^{c}	520	39	-63	58
	SF(0.1 M KCl)	CaM^{d}	240	41	-63	60
	NMR	$\text{TR}_1\text{C}^{\text{c}}$	430	39	-63	58
	SF	$\text{TR}_1\text{C}^{\text{d}}$	480	28	-99	58
	NMR(0.1 M KCl)	$\text{TR}_1\text{C}^{\text{c}}$	470	33	-84	58
	SF(0.1 M KCl)	$\text{TR}_1\text{C}^{\text{d}}$	280	24	-119	59
Slow	NMR	CaM^{a}	<40	51	-42	64
	SF	CaM^{b}	9.1	59	-29	68
	NMR(0.1 M KClO_4)	CaM^{c}	≈ 10	49	-63	68
	SF(0.1 M KCl)	CaM^{d}	24.0	64	-8	66
	SF	$\text{TR}_2\text{C}^{\text{d}}$	15.1	61	-17	66
	SF(0.1 M KCl)	$\text{TR}_2\text{C}^{\text{d}}$	37.2	52	-38	63

^aValues from Drakenberg et al. [13].

^bValues from Bayley et al. [31].

^cThis work.

^dValues from Martin et al. [26].

Figures

Figure 1. (A) The ^{43}Ca NMR line width as a function of the $[\text{Ca}^{2+}]/[\text{CaM}]$ ratio for a solution containing 1.2 mM CaM and 0.10 M KClO_4 at pH 7.4 and $T = 297$ K. Circles are experimental points. The solid curve is calculated assuming four binding sites per protein and that the metal ions exchange pairwise with the same rate. The quadrupole coupling constant was calculated to $\chi_e = 1.08$ MHz for all sites. (B) The temperature dependence of the ^{43}Ca NMR line width for a solution containing 1.2 mM CaM, 10 mM Ca^{2+} and 0.10 M KClO_4 at pH 7.4. Circles are experimental points. The solid curve is calculated assuming four binding sites per protein and the metal ions exchanging with pairwise identical rates, which results in: $\Delta H_f^\# = 39$ kJ/mol, $\Delta H_s^\# = 49$ kJ/mol, $\Delta S_{f,s}^\# = -63$ J/mol·K and $\chi_e = 1.08$ MHz. The dashed curve, two sites are exchanging: $\Delta H_f^\# = 39$ kJ/mol, $\Delta S_f^\# = -63$ J/mol·K and $\chi_e = 1.14$ MHz. The dotted curve, all four sites exchange with the same rate: $\Delta H^\# = 41$ kJ/mol, $\Delta S^\# = -63$ J/mol·K and $\chi_e = 0.94$ MHz.

The shown line width includes a line-broadening of 25 Hz.

Figure 2. (A) The line width of the ^{43}Ca NMR signal as a function of the $[\text{Ca}^{2+}]/[\text{TR}_1\text{C}]$ ratio for a solution containing 0.32 mM TR_1C at pH 7.0 and $T = 296$ K. Circles are experimental points. The solid curve is calculated assuming two identically exchanging sites: $\chi_e = 1.08$ MHz. (B) The ^{43}Ca NMR line width as a function of temperature for a solution containing 0.32 mM TR_1C and 1.0 mM Ca^{2+} at pH 7.0. Circles are experimental points. The solid curve is calculated with $\chi_e = 1.08$ MHz: $\Delta H_f^\# = 39$ kJ/mol and $\Delta S_f^\# = -63$ J/mol·K. The dashed curve, χ_e is allowed to vary: $\Delta H_f^\# = 39$ kJ/mol, $\Delta S_f^\# = -63$ J/mol·K and $\chi_e = 1.14$ MHz.

The shown line width includes a line-broadening of 50 Hz.

Figure 3. (A) The ^{43}Ca NMR linewidth as a function of the $[\text{Ca}^{2+}]/[\text{TR}_1\text{C}]$ ratio for a solution containing 0.77 mM TR_1C and 0.10 M KCl at pH 7.5 and $T = 296$ K. Circles are experimental points. The solid curve is calculated assuming two identically exchanging sites: $\chi_e = 1.08$ MHz. (B) The temperature dependence of the ^{43}Ca NMR line width for a solution containing 0.77 mM

TR₁C, 2.7 mM Ca²⁺ and 0.10 M KCl at pH 7.5. Circles are experimental points. The solid curve is calculated with $\chi_e = 1.08$ MHz: $\Delta H_f^\# = 33$ kJ/mol and $\Delta S_f^\# = -84$ J/mol·K. The dashed curve, χ_e is allowed to vary: $\Delta H_f^\# = 33$ kJ/mol, $\Delta S_f^\# = -84$ J/mol·K and $\chi_e = 1.17$ MHz. The shown line width includes a line-broadening of 25 Hz.

Figure 4. (A) The ⁴³Ca NMR spectrum of 0.75 mM TR₂C at different [Ca²⁺]/[TR₂C] ratios at pH 7.5 and T = 295 K. (B) Simulations using Lorentzian lines for the ⁴³Ca resonance for a sample containing 0.75 mM TR₂C and 2.0 mM Ca²⁺ at pH 7.5 and T = 295 K. From top to bottom: the measured spectrum, the simulated spectrum and the two Lorentzian lines (520 and 75 Hz wide and with a chemical shift difference between the lines of 230 Hz) that were used for the fitting.

Figure 5. (A) The partially relaxed ⁴³Ca NMR spectra of a 1.0 mM TR₂C solution containing 1.5 mM Ca²⁺ and 0.10 M KCl at pH 7.5 and T = 299 K. (B) The intensity of the resonance as a function of delay time. The solid curve represents the result of fitting the data to the equation

$$M_z^\tau = M_0 \{ 1 - [1 - k(1 - \exp(-0.5 \times 10^{-3}/T_1))] \exp(-\tau/T_1) \} \quad [19] \quad \text{giving } T_1 = 1.3 \times 10^{-3} \text{ s.}$$

Figure 1

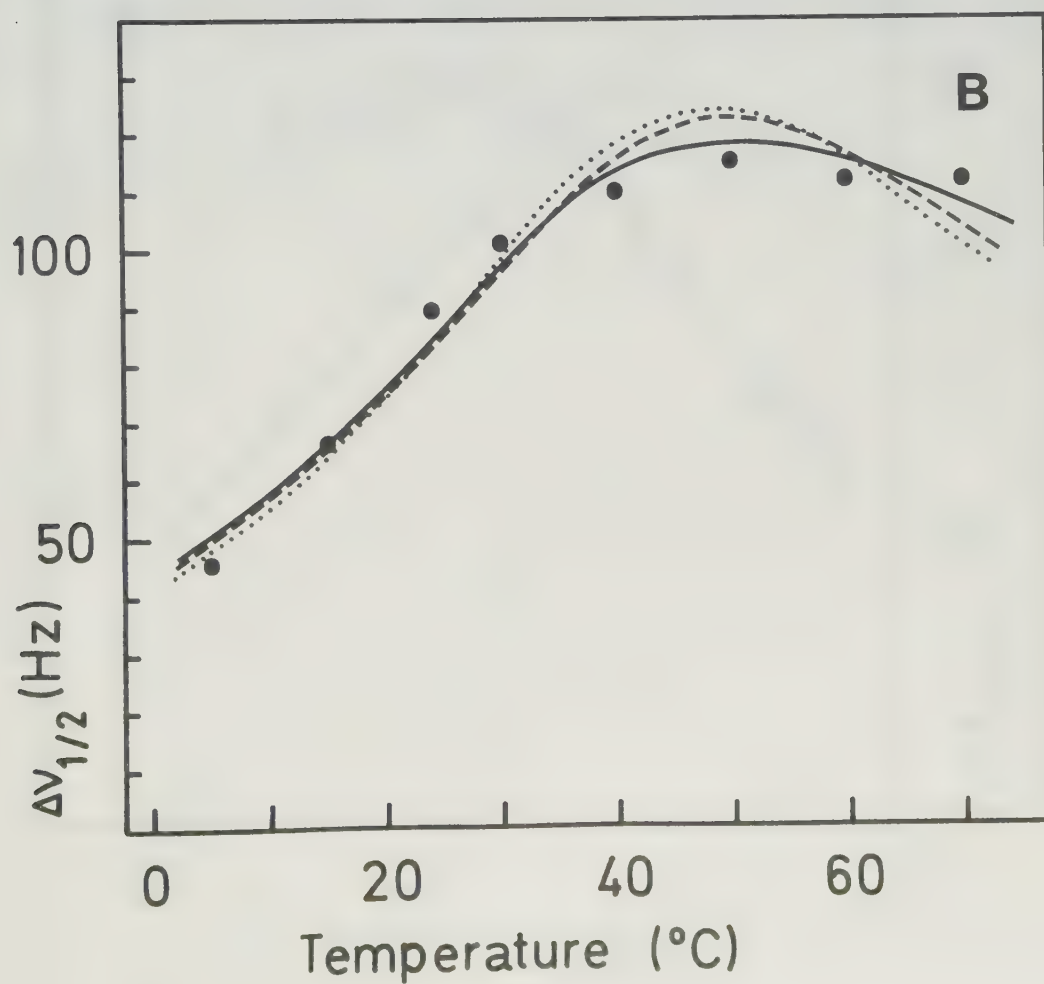
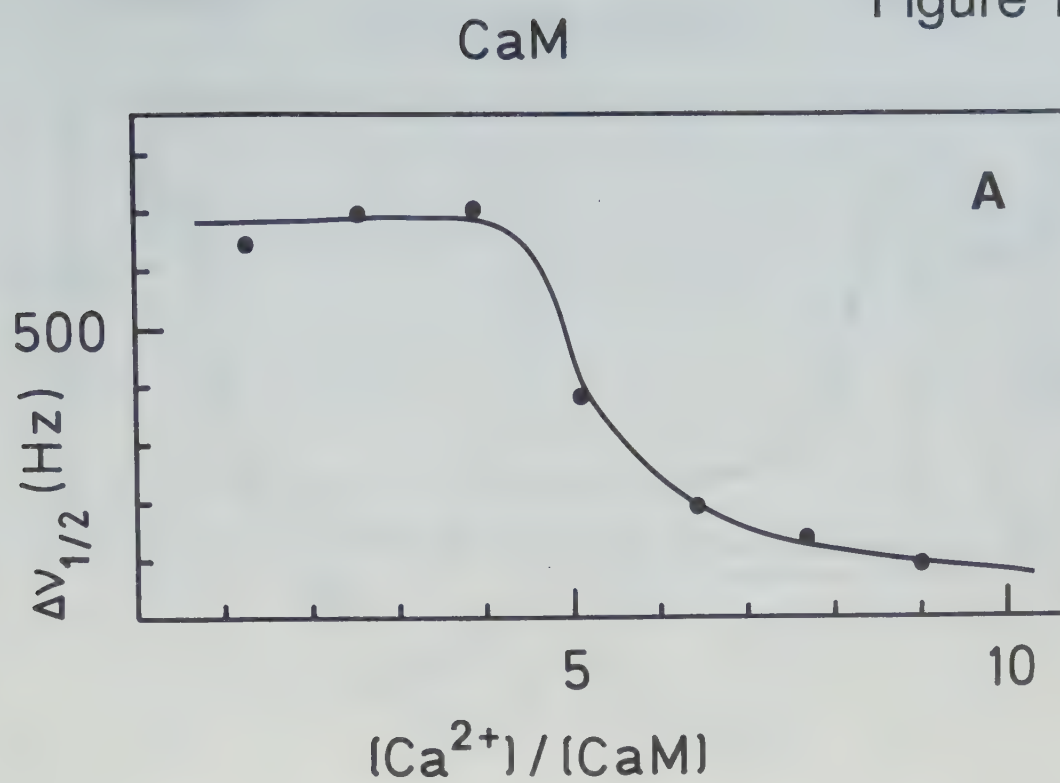


Figure 2

TR₁C (-KCl)

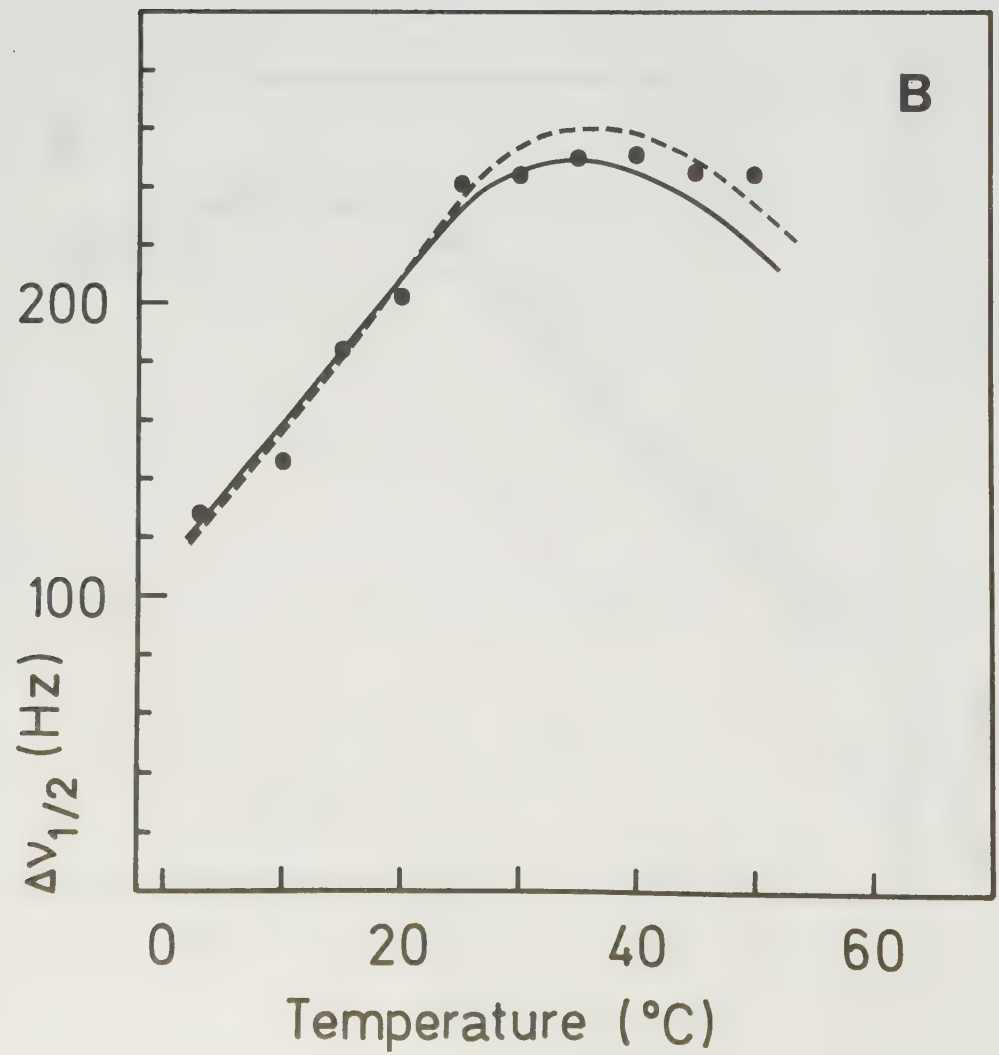
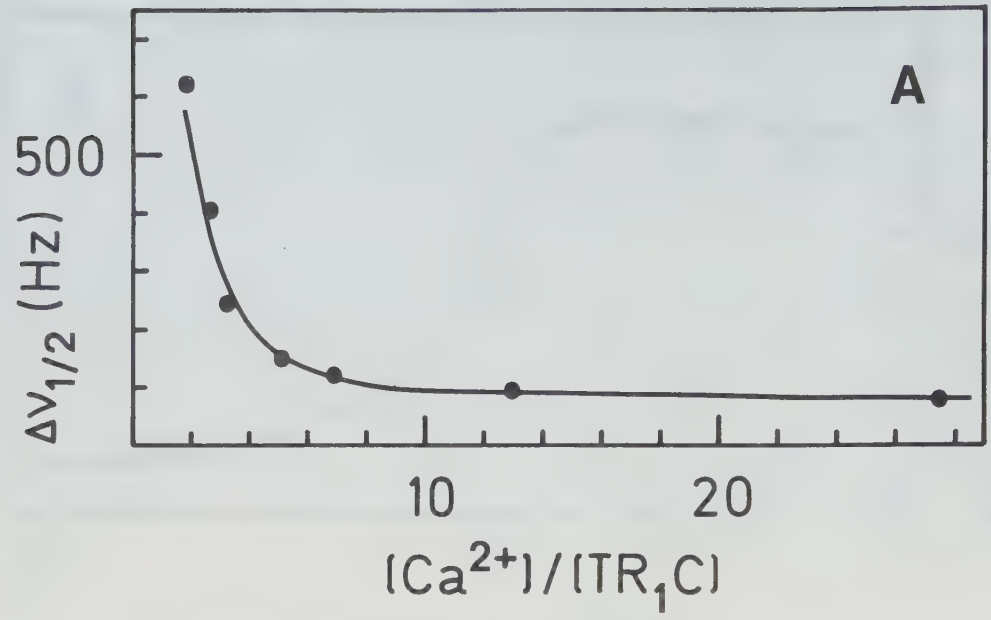


Figure 3

TR₁C (+KCl)

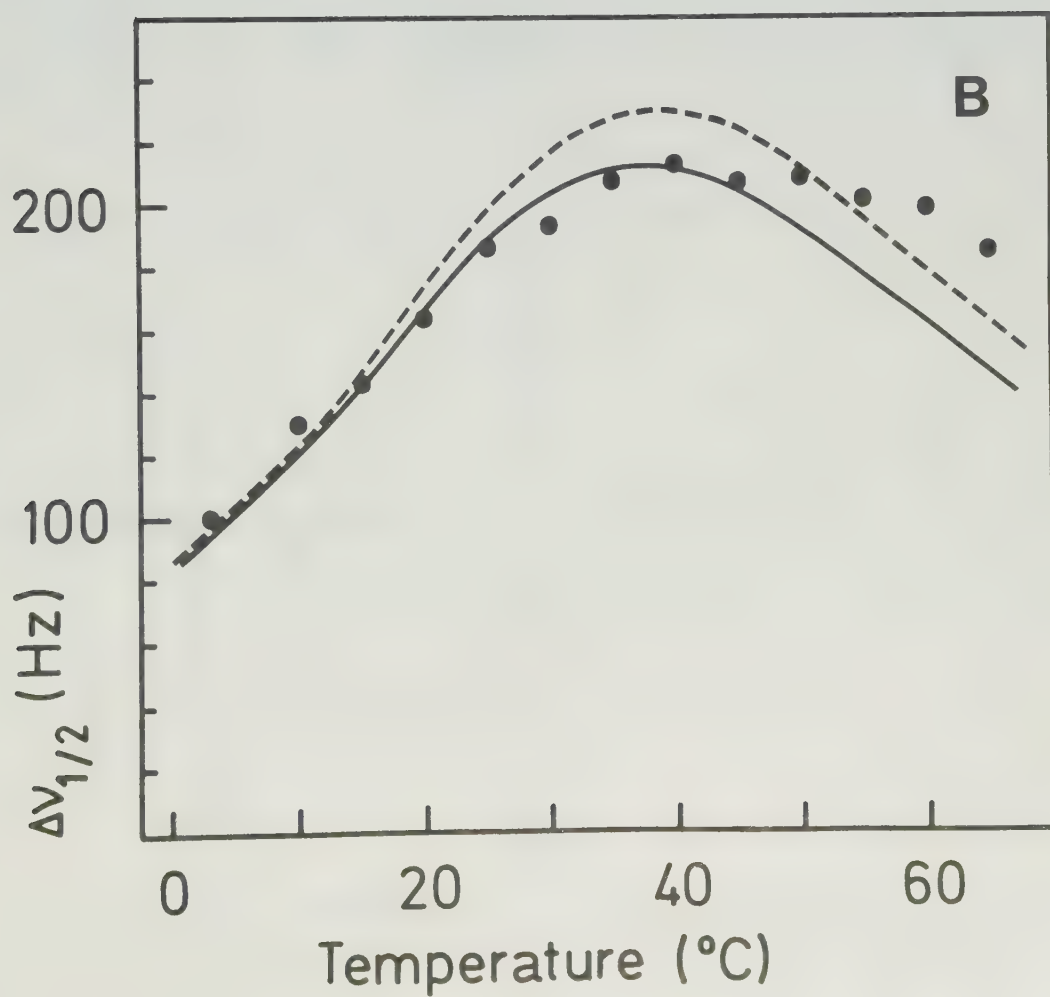
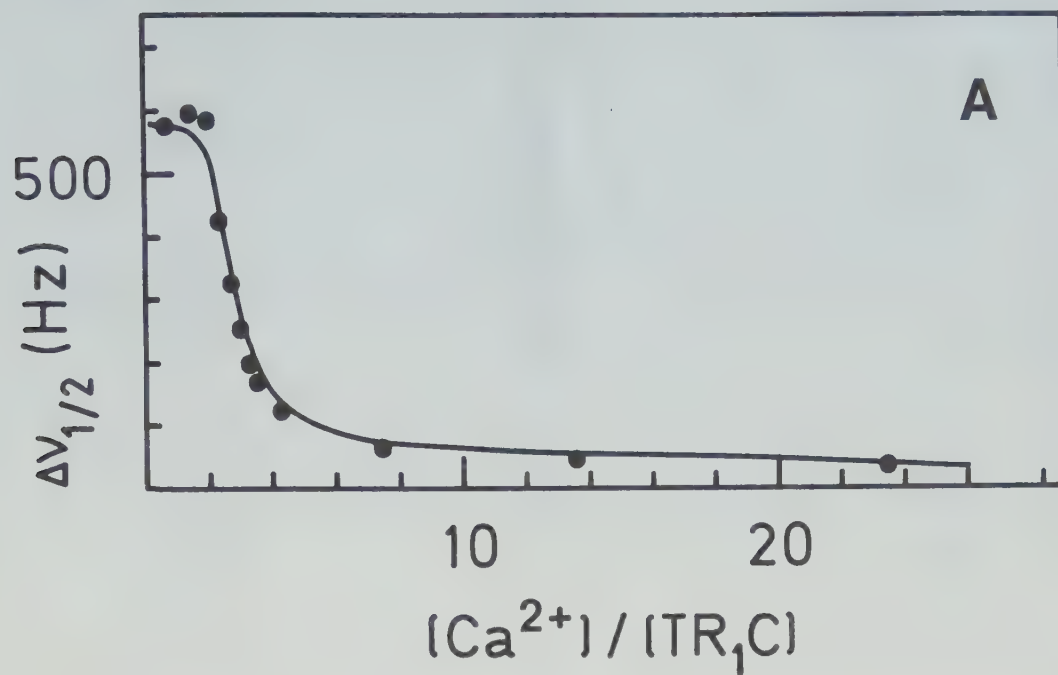


Figure 4

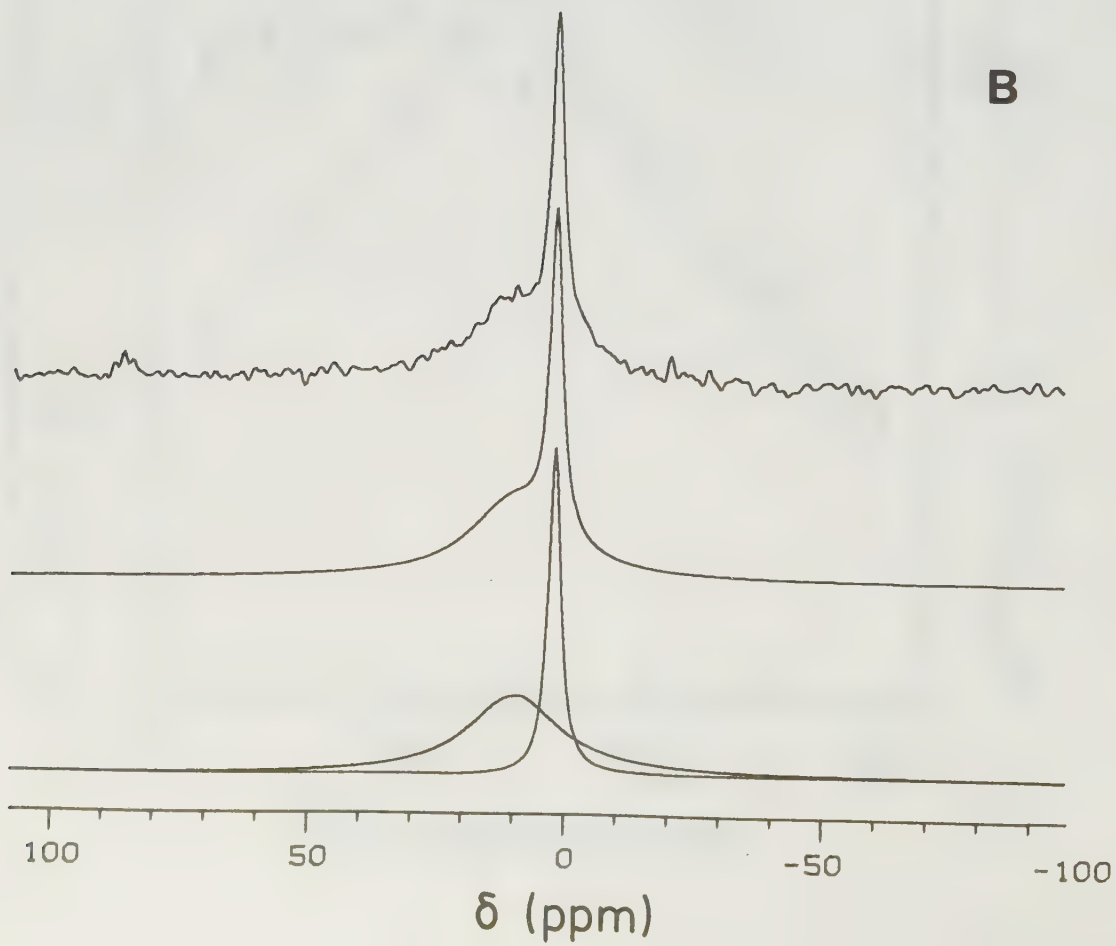
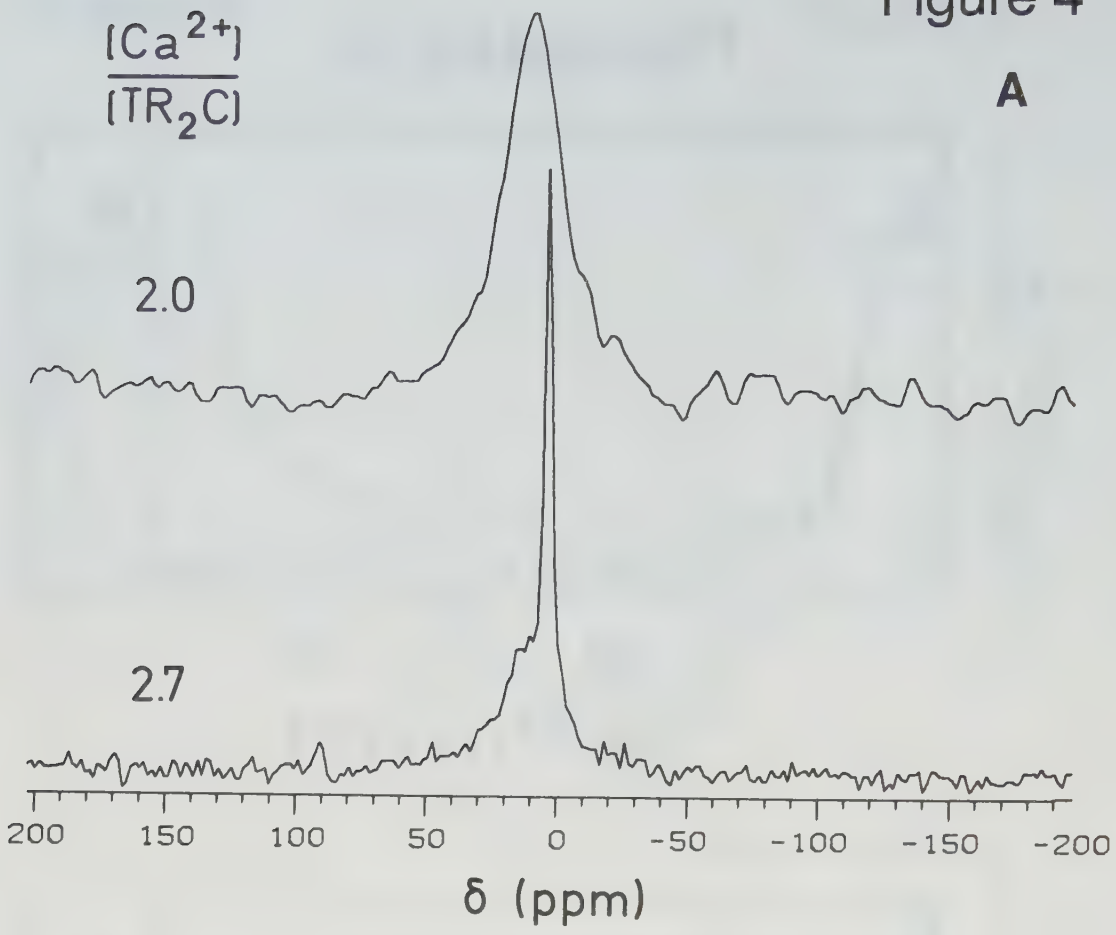
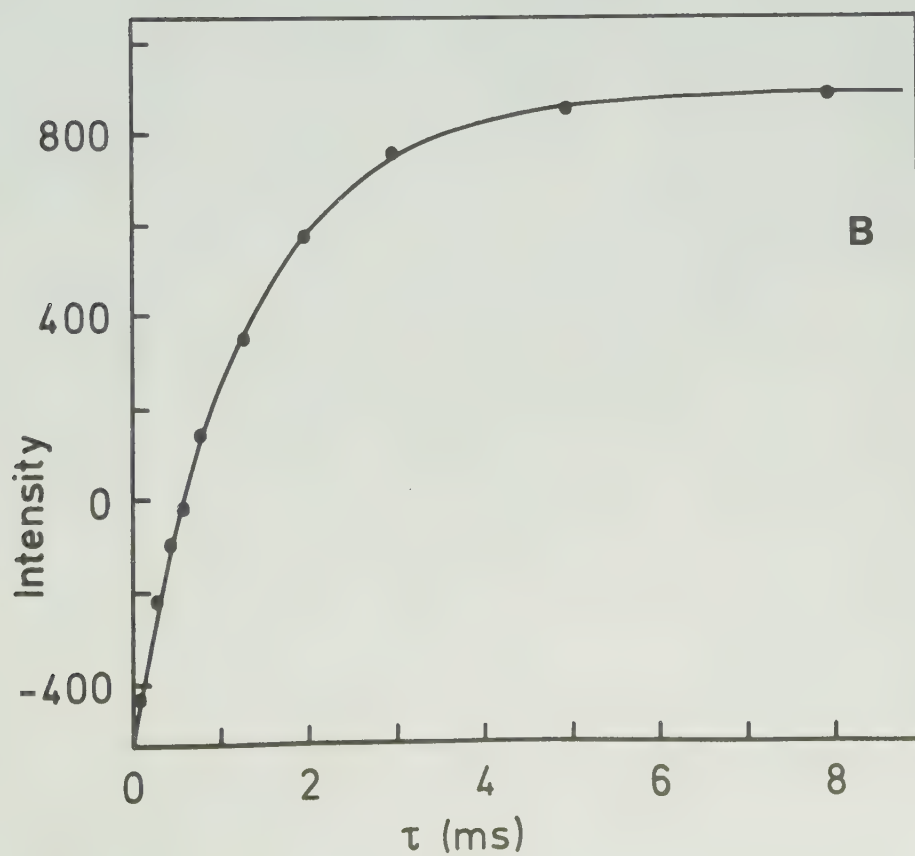
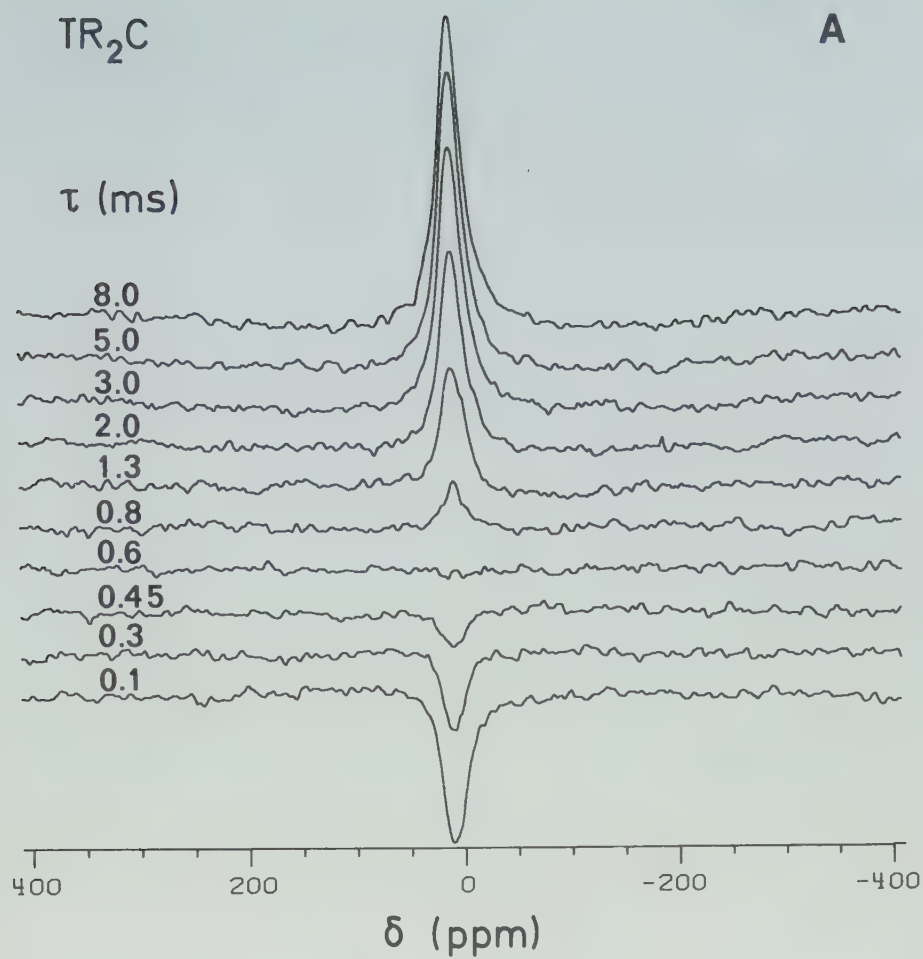


Figure 5



Paper V

A ^{113}Cd and ^1H NMR Study of the Interaction of Calmodulin with D600, Trifluoperazine and Some Other Hydrophobic Drugs

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The interaction of calmodulin with D600 (a methoxy derivative of verapamil), trifluoperazine and some other drugs was studied by ^{113}Cd and ^1H NMR. All four cation binding sites of calmodulin were found to be affected by the binding of the drugs to calmodulin. The physiologically active and inactive forms of felodipine were found to give qualitatively the same changes in the ^{113}Cd NMR spectra of calmodulin. The interpretation of this observation in terms of the physiological relevance of the binding to calmodulin is discussed. The binding constants for the two strongly bound trifluoperazine molecules were found to differ by one or two orders of magnitude.

A competition study showed that trifluoperazine replaces D600 from at least one binding site on calmodulin. Moreover the binding of D600 was found to be calcium dependent. The data indicate that two calcium ions bound to calmodulin are sufficient to render the binding site(s) accessible for D600.

Calmodulin (CaM) plays an important role in the Ca^{2+} -dependent regulation of eucaryotic cells [1–3]. The intracellular protein lacks tissue and species specificity; brain and testicular tissue are, however, rich sources [4]. It is a small, acidic protein ($M_r = 16\,700$, $\text{pI} = 3.9–4.3$) consisting of a single polypeptide chain, characterised by the absence of cysteine and tryptophan and the presence of the unusual amino acid trimethyllysine as well as a high phenylalanine/tyrosine ratio. Four calcium binding loops with surrounding α -helix structures have been identified from comparison of the sequence data of brain CaM and the known crystallographic structure of parvalbumin [5].

The binding of calcium to CaM creates a hydrophobic domain on the surface of the protein [6, 7] which appears to be essential for interaction of CaM with its receptor proteins. This domain is also considered to be involved in the binding of drugs. After the proposal by Levin and Weiss [8, 9] that the pharmacological action of antipsychotic tranquilizers of the phenothiazine type is mediated through their interaction with CaM, the inhibition of the biological activity of CaM by drugs has been extensively studied.

In the study of calcium binding proteins, metal NMR studies and high-resolution ^1H -NMR studies have been shown to be useful (for reviews see [10, 11]). In the present paper ^{113}Cd NMR has been employed as a tool to examine the interaction of D600 (a methoxy derivative of verapamil, the original drug used as a coronary vasodilator), trifluoperazine, the fluorescence probe TNS and several other substances with CaM. Various studies have shown that the Cd^{2+} ion is a good replacement for Ca^{2+} in the study of calcium binding proteins

[11]. In addition, the interaction of D600 with CaM has been studied by ^1H NMR as a function of the Ca^{2+} ion content of CaM. The five methoxy groups of D600 can be readily observed and provide sensitive monitors of the ability of this drug to bind to CaM. Further, a competition study between D600 and TFP will be described.

MATERIALS AND METHODS

Bovine testes calmodulin was purified essentially by the procedures described by Dedman et al. [12] and Jamieson and Vanaman [13]. For certain preparations additional chromatography on DEAE-Sephadex A-25 was necessary to purify the protein. The purity of CaM was confirmed by agarose gel electrophoresis and ultraviolet-absorbance and finally by electrophoresis in polyacrylamide/sodium dodecyl sulphate gel.

Calcium-free CaM (<0.2 mol Ca^{2+} /mol CaM) was obtained by passing an aqueous protein solution through a column of Na^+ -saturated Chelex-100. Afterwards the solution was lyophilized and dissolved in double distilled water or $^2\text{H}_2\text{O}$.

An aqueous 0.10 M $\text{Cd}(\text{ClO}_4)_2$ solution was prepared by dissolving CdO (96% isotope enriched in ^{113}Cd , Oak Ridge National Laboratory, Oak Ridge, TN) in HClO_4 . The solution was adjusted to pH 6.2 by adding 1.0 M Tris to a final concentration of 0.15 M.

TFP and D600 were obtained from Smith, Kline and French Labs (Philadelphia, PA) and Knoll AG, FRG, respectively. TNS was purchased from Eastman Kodak Co. (Rochester, NY). Oxidized felodipine was a gift from AB Hässle Research Laboratories (Mölnådal, Sweden). Chremaphor RH 410 was obtained from BASF, FRG. The chemicals were used without further purification.

The ^{113}Cd NMR spectra were recorded at 56.55 MHz on the homebuilt UL 6-T Fourier-transform spectrometer [14]. Typical spectra were obtained by using 7.5 - μs pulses ($\approx 45^\circ$), a spectral width of $\pm 10\,000$ Hz, an acquisition time of 0.06 s and a waiting time of 0.5 s. The temperature was 23°C . Chemical

Abbreviations. CaM, calmodulin; D600, $\alpha\{3-[2-(3,4\text{-dimethoxyphenyl})\text{-ethyl}]\text{-methylamino}\}$ propyl-3,4,5-trimethoxy- α -(1-methylethyl)-benzene-acetonitrile; TFP, trifluoperazine; TNS, 6-*p*-toluidino-2-naphthalene sulfonic acid; $\text{CaM} \cdot \text{Cd}_4$, cadmium-loaded calmodulin; $\text{CaM} \cdot \text{Ca}_4$, calcium-loaded calmodulin; W-7, *N*-(6-aminoethyl)-5-chloro-1-naphthalene-sulfonamide; ANS, 8-anilino-naphthalene-sulfonate; felodipine, 4-(2,3-dichlorophenyl)-1,4-dihydropyridine-2,6-dimethyl 3,5-dicarboxylic 3-ethyl ester and 5-methyl ester; chremaphor RH 410, ethoxy ricinoleic acid.

shifts were measured relative to an aqueous 0.1 M solution of $\text{Cd}(\text{ClO}_4)_2$. Shifts to low field are taken as positive. Each spectrum is the sum of 25000 free induction decays (FID). The signal-to-noise ratio was improved by an exponential multiplication of the FID which gives rise to a line broadening of ≈ 30 Hz.

Some ^{113}Cd NMR spectra were recorded at 80.24 MHz on a Nicolet 360 WB NMR spectrometer with corresponding spectral acquisition parameters to those used at 56.55 MHz. Also on the Nicolet spectrometer a homebuilt probe for horizontal sample orientation was used.

Proton spectra were obtained at 361.79 MHz on a Nicolet 360 WB NMR spectrometer using 5-mm NMR tubes containing about 0.5 ml of solution. Typical acquisition parameters were a 9.0- μs pulse ($\approx 90^\circ$), a spectral width of ± 2000 Hz and an acquisition time of 1.02 s. The residual H_2O peak was suppressed by a selective inversion sequence: $180^\circ\text{-}\tau_1\text{-}90^\circ\text{-}\tau_2$. The 180° pulse was a soft (5-ms long) pulse on the H_2O resonance. The waiting time, τ_1 , was adjusted to allow the H_2O resonance to relax to $M_z = 0$. After that the normal 90° pulse was applied for observing the protein spectrum. Each spectrum is the sum of 500 free induction decays. The resolution was enhanced by a double exponential multiplication. Spectra were obtained at ambient temperature, 23°C , and chemical shifts are reported from an internal standard of 2,2-dimethyl-2-silapentane-5-sulfonate.

pH values were measured with a Radiometer PHM 63 Digital pH meter equipped with a Schott Mainz pH combination glass electrode N60-EE. The pH values reported for ^1H NMR are not corrected for deuterium isotope effects.

RESULTS

^{113}Cd NMR

In the absence of drugs two ^{113}Cd NMR signals are observed, one (A) at -87.6 ppm and the other (B) at -114.6 ppm (cf. Fig. 2, top left), which have been assigned to the two stronger, slowly exchanging sites [15].

Addition of aliquots of an aqueous TFP solution (50 mM) to a sample containing 1.39 mM $\text{CaM} \cdot \text{Cd}_4$ (pH 7.0) largely affects the metal binding sites as inferred from the ^{113}Cd NMR spectra (see also [15]). Already at 0.1 mol TFP/mol $\text{CaM} \cdot \text{Cd}_4$ considerable effects can be observed in the ^{113}Cd NMR spectra (Fig. 1). The ^{113}Cd NMR chemical shifts of both signals are affected by the addition of TFP. Signal A has shifted about 11 ppm to lower frequency after the addition of 1 equivalent of TFP and stays almost constant when more TFP is added. Signal B is shifted much less about 1.5 ppm due to the first equivalent of TFP and shifts an additional 2.5 ppm due to the addition of the second equivalent of TFP. Furthermore, two new signals appear and become most clearly visible at a TFP: $\text{CaM} \cdot \text{Cd}_4$ ratio of 2:1 (Table 1, and Fig. 2b).

A solution of D600 (55 mM) dissolved in ethanol was successively added to a 1.2 mM $\text{CaM} \cdot \text{Cd}_4$ solution (pH 7.6). D600 induced changes in the environments of the four bound cadmium ions, as can be observed in the ^{113}Cd NMR spectra (Fig. 2a). When the ratio of D600 to $\text{CaM} \cdot \text{Cd}_4$ is increased the chemical shifts for signals A and B are continuously altered to higher field (Fig. 1). The intensity of signal A is somewhat decreased, probably due to an increased T_1 . An increase in line width is observed for signal A from 100 Hz with no added D600 to a maximum of 145 Hz at 0.5 mol D600/mol CaM . A broad resonance around -100 ppm is appearing when the ratio of D600 to $\text{CaM} \cdot \text{Cd}_4$ is 1:1. This signal becomes sharper during

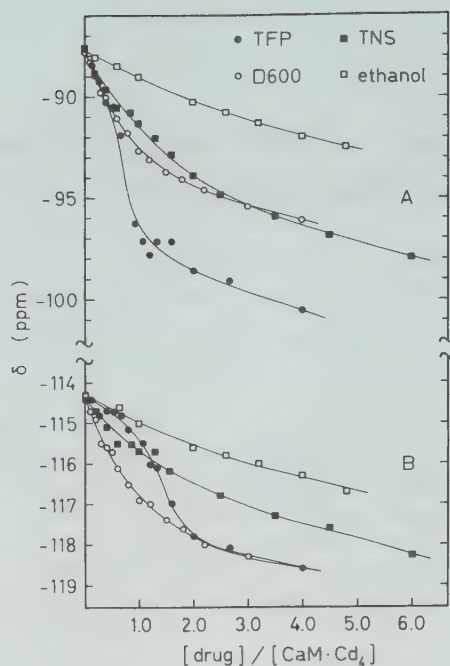


Fig. 1. The chemical shifts of signals A and B during the titrations of TFP, D600, TNS, and ethanol to cadmium-ion-loaded calmodulin

Table 1. The ^{113}Cd NMR chemical shifts for all the observed signals in the ^{113}Cd NMR spectra of cadmium-loaded calmodulin when drugs are added. The final values are taken for the different titrations. The ^{113}Cd NMR chemical shifts are relative to a standard solution of $\text{Cd}(\text{ClO}_4)_2$ in water. The chemical shifts of TFP are recalculated from an earlier report [15] by adding 0.6 ppm to the reference to obtain the same conditions as for the other drugs

Drug	[Drug]	δ			
	[CaM · Cd ₄]	signal A		signal B	
		ppm			
None	0	−87.6	—	—	−114.6
TFP	2.0	−99.1	−112.9	−115.2	−118.1
D600	4.0	−96.1	−112.6	−116.3	−118.5
TNS	6.0	−98.0	−112.0	−119.9	−118.3
Felodipine (red)	3.0	−91.4	—	—	−115.9
Felodipine (ox)	3.0	−94.5	−110	—	−117.0
Ethanol	4.8 ^a	−92.5	−100	—	−116.7
FMN	2.0	−88.0	—	—	−114.9

^a This value corresponds to the amount of ethanol that is added as solvent when 4.8 mol (D600 or TNS)/mol CaM are added.

the titration. When four equivalents of D600 are added, the broad resonance has changed to two sharp signals, analogous to those observed for a TFP: CaM ratio of 2:1 (Table 1). The intensities of these new resonances are comparable to those of signals A and B.

Addition of TNS (28 mM) dissolved in 50% ethanol to a solution of 1.1 mM $\text{CaM} \cdot \text{Cd}_4$ (pH 7.5) also resulted in changes in the ^{113}Cd NMR spectra. The signals A and B were continuously moving to higher field during the titration (Fig. 1). For signal A the intensity decreased while the line

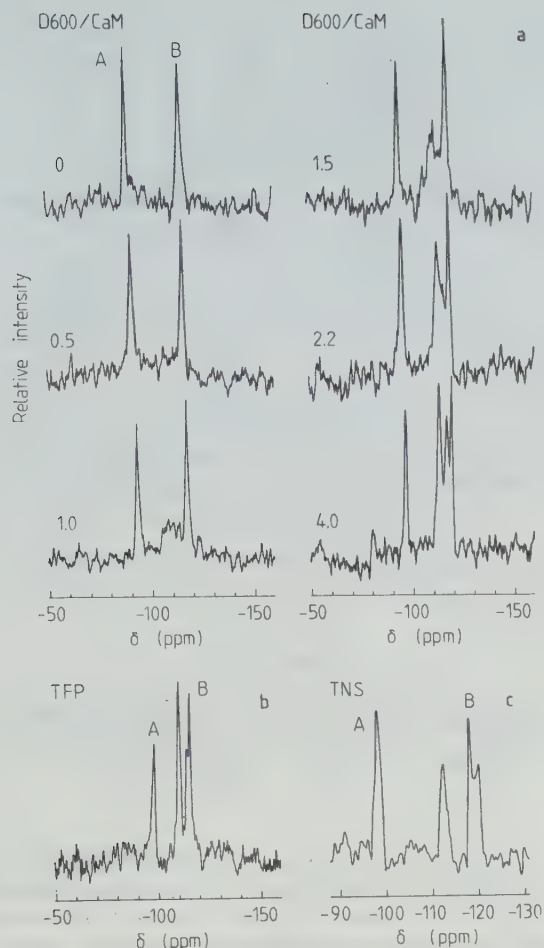


Fig. 2. The ^{113}Cd NMR spectra of different drugs. (a) 2.5 ml 1.2 mM $\text{CaM} \cdot \text{Cd}_4$, pH 7.6 as a function of added 55 mM D600 dissolved in ethanol. (b) 1.4 mM $\text{CaM} \cdot \text{Cd}_4$, 2.8 mM TFP, pH 7.0. (c) 1.1 mM $\text{CaM} \cdot \text{Cd}_4$ 6.6 mM TNS, pH 7.5. The spectra in (a) and (b) were obtained at 56.55 MHz, while the spectrum in (c) was obtained at 80.24 MHz

width was unaffected by the addition of TNS. For signal B the intensity remained the same while the line width increased from 105 Hz to 155 Hz (from 0 to 6.0 mol TNS/mol $\text{CaM} \cdot \text{Cd}_4$). This broadening is caused by the overlap of this resonance with one from the two more weakly bound cadmium ions. Only when the ratio of TNS to $\text{CaM} \cdot \text{Cd}_4$ is 2:1 or more can a broad signal be observed around -100 ppm for one of the two weaker sites. The signal narrows as the amount of TNS is increased. When the molar ratio of TNS to $\text{CaM} \cdot \text{Cd}_4$ is 6:1 three separated signals could be observed. In a spectrum obtained at 80.24 MHz it was possible to resolve the signals of the four protein-bound cadmium ions (see Table 1 and Fig. 2c).

An aqueous solution of FMN (65 mM), a water-soluble flavin dye, was successively added to a 1.3 mM $\text{CaM} \cdot \text{Cd}_4$ solution (pH 7.8). No alterations in chemical shifts or line widths of signals A and B could be observed in the ^{113}Cd NMR spectrum (Table 1).

A 100 mM solution of oxidized felodipine dissolved in a mixture of ethanol/chremaphor RH410 (volume ratio 9:1) was added to $\text{CaM} \cdot \text{Cd}_4$ (1.2 mM $\text{CaM} \cdot \text{Cd}_4$ pH 8.2). The alterations in chemical shifts of signals A and B are larger than in the case of felodipine (Table 1) [16]. Moreover, a third signal at -110 ppm is observed when the ratio of oxidized felodipine to CaM is 3:1.

Since neither D600 nor TNS is soluble in water to concentrations required for these studies, both were dissolved in ethanol solutions. As a check of the solvent effect on calmodulin, the same amount of ethanol was added to a solution of 1.1 mM $\text{CaM} \cdot \text{Cd}_4$ (pH 7.5) as during the titrations with D600 and TNS. The ethanol affected the ^{113}Cd NMR chemical shift for signals A and B (Fig. 1): a broad signal at about -100 ppm could be seen when the ethanol concentration was 6% (v/v). This corresponds to the amount of ethanol added to reach a molar ratio of D600 to CaM of 3:1. A similar control was made with the ethanol/chremaphor mixture used to dissolve felodipine. This mixture affected the ^{113}Cd spectrum in the same way as pure ethanol. For a solution with a TNS to CaM ratio of 4.5:1 an additional 75 μl of ethanol was added. This was calculated to correspond to the amount of ethanol which is present as solvent at a ratio of 1.5 for TNS to CaM. The ^{113}Cd NMR spectrum remained the same as before within experimental error. This indicates that the slight change in shift of both signals A and B at drug to CaM ratios above 2 is not due to variations in solvent but caused by the drug binding to weak binding sites.

^1H NMR

The proton NMR spectrum of D600 contains a few signals that are well-suited for the study of the binding of D600 to CaM. These are the five methoxy groups, which give rise to four signals (intensities 2:1:1:1) with chemical shifts between 3.7 ppm and 3.9 ppm, the *N*-methyl group at 2.75 ppm and the aromatic protons (Fig. 3a). All the methyl groups resonate in regions of the spectrum where there are very few protein signals, whereas the aromatic proton resonances will overlap with those from phenylalanines and tyrosines from the protein.

When D600 [140 mM D600 dissolved in ($^2\text{H}_6$) ethanol] is added to a solution of 2 mM metal-free CaM at pH 7.5 only minor changes are observed in the ^1H NMR spectrum of either CaM or D600 (Fig. 3b). The most pronounced effect is on a methionine resonance of CaM that shifts from 2.09 ppm to 2.01 ppm at a D600 to CaM ratio of 2:1. The solution has a tendency to precipitate when 2 mol D600/mol CaM is added. As the ratio of D600 to CaM is increased the solution becomes more turbid. When Ca^{2+} ions are added to the CaM D600 solution dramatic effects can be observed in the spectrum of D600 as well as CaM. Furthermore the turbidity disappears.

The binding of calcium ions to CaM in the presence of D600 resulted, as with drug-free CaM [17, 18], in changes in the resolved resonances attributed to tyrosine-138, histidine-107, trimethyllysine-115 and the high-field-shifted methyl groups in the region of -0.2 ppm to $+0.7$ ppm. The effect on the D600 spectrum is most clearly seen for the methoxy resonances (Fig. 3c and 4). The two signals due to the groups bound to ring I of D600 both split into two resonances and shift from 3.83 ppm to 3.64 ppm and 3.73 ppm and from 3.70 ppm to 3.67 ppm and 3.72 ppm (see Fig. 4). The two signals from ring II methoxy groups also shift from 3.83 ppm to 3.66 ppm and from 3.78 ppm to 3.73 ppm. However, the latter two signals never split up. The signal from the *N*-Me group at 2.75 ppm also shifts and splits into two signals (not shown) upon the addition of calcium ions.

The overlap between signals from D600 and CaM in the aromatic region of the spectrum renders a detailed interpretation of the shifts observed upon Ca^{2+} addition difficult. However, in the phenylalanine region there are some shift changes on calcium addition which are not the same as in the drug-free solution.

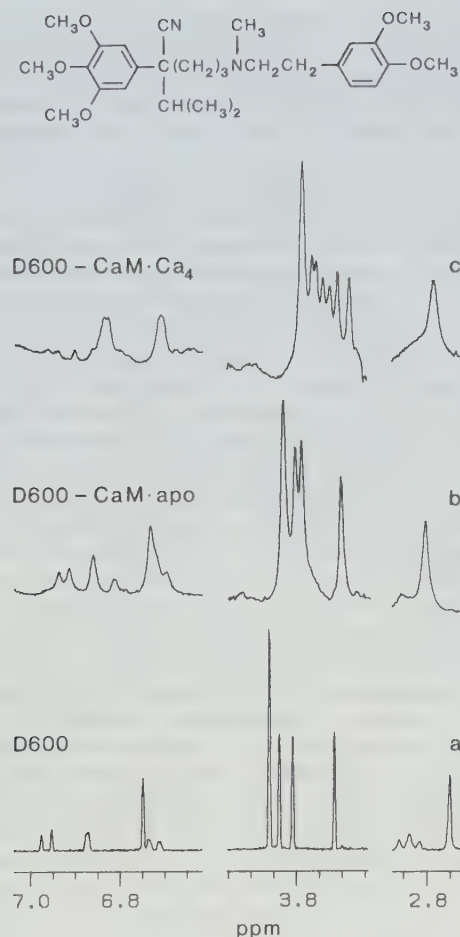


Fig. 3. The aromatic resonances and the signals of the methoxy groups of D600, ^1H NMR spectra. (a) 8 mM D600 in 50% ($^2\text{H}_6$) ethanol; (b) 8 mM D600, 2 mM calcium-free calmodulin, pH 7.5; (c) addition of calcium to a concentration of 8 mM to the sample in b. The molecular structure of D600 is shown above the spectra

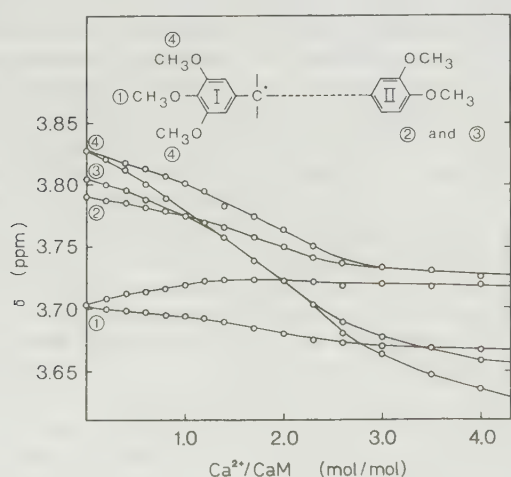


Fig. 4. Alterations in the chemical shifts of the methoxy groups of D600 as calcium ions are added, 2 mM CaM, 4 mM D600, pH 7.5

When D600 [140 mM D600 dissolved in ($^2\text{H}_6$) ethanol] is added to a solution containing 2 mM CaM $\cdot$ Ca₄ (0.2 M KCl, pH 7.0) large changes of the ^1H NMR signals representing CaM $\cdot$ Ca₄ as well as D600 are observed. The changes in the

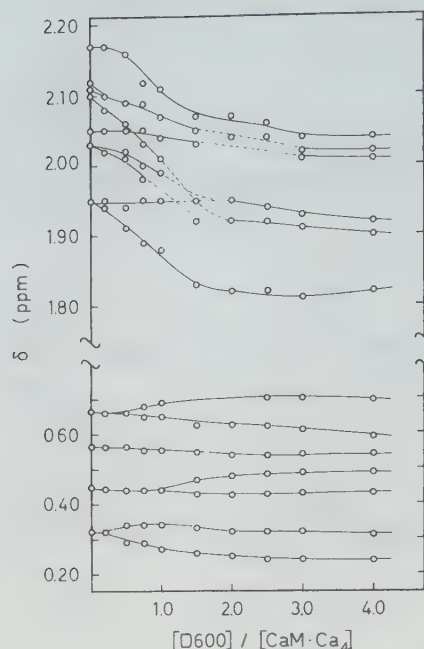


Fig. 5. Changes in chemical shifts in ^1H NMR spectra of some resonances in the aliphatic region during D600 titration. The chemical shifts were taken from the same D600 titration as depicted in Fig. 4

CaM $\cdot$ Ca₄ spectrum are similar to those observed on addition of TFP [18, 19]. Several signals in the methionine region, 1.8–2.2 ppm, shift upfield by up to 0.2 ppm (Fig. 5). These effects seem to level off at a D600/CaM ratio of 2:1. Some further effects are detectable in the region of high-field-shifted methyl groups between 0.2–0.9 ppm, not very pronounced and therefore difficult to interpret (Fig. 5).

In the aromatic region (Fig. 6), the resonances assigned to histidine-107 [17] are shifted from 7.77 ppm to 7.85 ppm and from 6.97 ppm to 6.92 ppm for the C2 and the C4 protons, respectively, after addition of 1 equivalent of D600. In parallel with these changes the resonances from some phenylalanines between 7.12 ppm and 7.45 ppm also change to become a broad, ill-resolved feature (Fig. 6). Interestingly, the resonances assigned to the two tyrosines (Tyr-99 and Tyr-138) seem to be unaffected by the addition of D600 as judged from the resonances at 6.35 ppm, 6.56 ppm and 6.77 ppm (see Fig. 6.)

As mentioned above, the D600 methoxy and *N*-methyl resonances appear in regions reasonably free of protein resonances and can therefore easily be observed. The *N*-methyl signal grows up as a doublet at 2.82 ppm with increasing D600 concentration. When 2.5 equivalents of D600 are added these two signals have merged. At low concentrations of D600 the methoxy signals start out as a rather complex pattern giving rise to more signals than can be assigned to the four different methoxy groups. At most six resonances can be observed. With increasing D600 concentrations these signals merge and move towards the positions for free D600.

To a solution consisting of 2 mM CaM $\cdot$ Ca₄ and 4 mM D600, pH 7.5, increasing amounts of TFP were added, resulting in the displacement of the methoxy resonances towards the chemical shifts of the methoxy groups of free D600 (Fig. 7). The alterations resemble very much those that are observed when Ca²⁺ is added to a solution containing both metal-free CaM and D600 but appear in reverse order (compare Fig. 4 and 7). This is especially seen for the signals starting

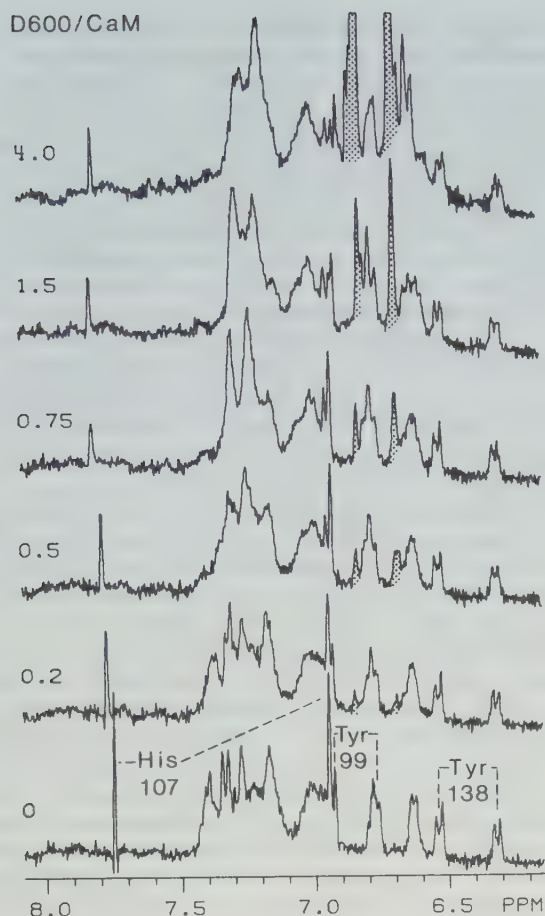


Fig. 6. Aromatic region of the ^1H NMR spectra of calcium-loaded calmodulin as a function of added D600, 2 mM CaM, 0.2 M KCl, pH 7.0. The ratio of total D600/total protein is indicated for each spectrum. The signals of D600 are shaded

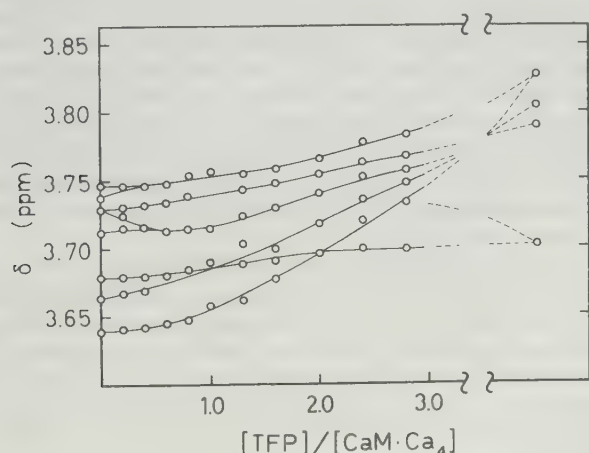


Fig. 7. Effects of TFP on the chemical shifts of the methoxy groups of D600, 2 mM CaM, 8 mM Ca^{2+} , 4 mM D600, pH 7.5. The shift values of the methoxy groups of free D600 in the presence of calcium-free calmodulin are indicated to the right. The dashed lines are based on Fig. 4

at 3.640 ppm, 3.663 ppm and 3.680 ppm. These move to chemical shifts very near those when 2.3 equivalents of Ca^{2+} have been added in the Ca^{2+} titration. The complexity of the aromatic region due to overlapping signals from TFP, protein and D600 renders it impossible to follow the resonances of D600.

DISCUSSION

The use of cadmium ions as a probe in the study of calcium binding proteins has been reported for various proteins (for review see [11]). Resolved resonances have been observed in the study of proteins of the calmodulin family, parvalbumin, troponin C and calmodulin [15, 20, 21]. Replacement of calcium ions by cadmium ions gives rise to virtually identical ^1H NMR spectra for the metal-saturated protein [22] (and Andersson et al., unpublished). Also, the sequence of binding of the metal ions to the individual sites in the apoprotein seems to be the same as monitored by tyrosine-99, tyrosine-138, histidine-107, trimethyllysine-115 and the phenylalanines in ^1H NMR spectra.

The ^{113}Cd NMR spectrum of fully cadmium-loaded calmodulin ($\text{CaM} \cdot \text{Cd}_4$) shows only two signals, -88 ppm (signal A) and -114.5 ppm (signal B), respectively (Fig. 2a, top left). Those signals are attributed to the two most strongly bound Cd^{2+} ions. Signals from the other two bound cadmium ions are normally not observed, probably due to severe exchange broadening between protein-bound and free metal ions [15]. Our studies here have shown that all four cation binding sites are affected by the binding of the studied substances (FMN being the only exception). The ^{113}Cd NMR shift changes reported in Fig. 1 indicate that the binding constants for the two strongly bound TFP molecules differ by one or two orders of magnitude.

The binding of the first molecule TFP to metal-ion-loaded CaM causes a conformational change in the protein that gives rise to an 11-ppm upfield shift of signal A. It is therefore tempting to assume that this TFP molecule is bound close to the metal ion binding site giving rise to signal A. However, since there appears to be an overall conformational change of the protein upon TFP binding it is presently not possible to define the binding sites. Although it is known that resonance A represents one of the two strongly bound metal ions [15], there is as yet no definite assignment of site A to any of the four cadmium binding loops, numbered I to IV from the NH_2 terminus, that have been identified in the CaM molecule. Work on such an assignment is in progress in our laboratory. Site B is affected by both the first and the second TFP molecule added, the ^{113}Cd chemical shift undergoing a larger change upon addition of the second TFP molecule. The TFP binding to CaM also affects the two weaker binding sites. However, since the ^{113}Cd NMR signals from these sites are not observable when no TFP is present, it is impossible to estimate how large the chemical shift changes are. The effect is instead readily apparent as a slowing down of the metal ion exchange rate for these two ions, presumably a reflection of increasing binding constants. The further small changes that occur for the chemical shifts of signal A and B after the addition of 2 equivalents of TFP may reflect the binding of additional TFP molecules. This would agree with the report by Levin and Weiss [9] that two TFP molecules bind to CaM in a calcium-dependent manner with a binding constant of 10^6 M^{-1} and that additional molecules bind in a calcium independent manner with a binding constant of $2 \times 10^2 \text{ M}^{-1}$.

For D600 and TNS we find the same overall effects as discussed above for TFP in the ^{113}Cd NMR spectra of $\text{CaM} \cdot \text{Cd}_4$. That is, the resonances of the two high-affinity cation sites change to lower chemical shifts and the exchange rates between bound and 'free' cadmium ions decrease for the two low-affinity sites, although the effects are not as dramatic with these two compounds as with TFP. The three substances bound to $\text{CaM} \cdot \text{Cd}_4$ result in somewhat different chemical

shifts for the four Cd^{2+} ions in the ^{113}Cd NMR spectra (Table 1). This probably reflects the differences in the protein conformation changes which they induce. Different studies with a series of homologous parvalbumins have indicated that ^{113}Cd chemical shifts are very sensitive to small changes in the overall structure [23].

The number of 'strong' binding sites for D600 and TNS to $\text{CaM} \cdot \text{Cd}_4$ cannot be directly obtained from the changes in the chemical shifts in the ^{113}Cd NMR spectra in the same way as for TFP. An analysis of the curves in Fig. 1 assuming one strong binding site gives a binding constant of D600 to CaM of $10^3 - 10^4 \text{ M}^{-1}$. Different numbers of binding sites for different drugs have been reported: three strong binding sites for W-7 ($K_b = 9.1 \times 10^4 \text{ M}^{-1}$) [24], three strong binding sites for chlorpromazine ($K_b = 2 \times 10^5 \text{ M}^{-1}$) [25] and two strong binding sites for amitriptyline ($K_{b1} = 5.6 \times 10^4 \text{ M}^{-1}$ and $K_{b2} = 4.3 \times 10^4 \text{ M}^{-1}$) [26].

The observed maximum line broadening of the ^{113}Cd NMR signal A in the D600 titration occurs at a D600/ $\text{CaM} \cdot \text{Cd}_4$ molar ratio of about 0.5. This is similar to but less pronounced than we have previously observed for the TFP binding to CaM [15]. The line broadening was in the latter case explained as due to chemical exchange of TFP molecules between CaM molecules and the rate of the conformational change giving rise to the change in chemical shifts of signal A could be estimated to be about 10^3 s^{-1} . Similarly we can now estimate an exchange rate of about $2.5 \times 10^3 \text{ s}^{-1}$ in the D600- $\text{CaM} \cdot \text{Cd}_4$ case. No line broadening is observed in the TNS case.

The unchanged ^{113}Cd NMR spectra of $\text{CaM} \cdot \text{Cd}_4$ upon addition of FMN indicate that this water-soluble flavin dye does not bind to CaM , or is incapable of inducing a conformational change in calmodulin. FMN, like CaM , is negatively charged, while TFP is positively charged. However, LaPorte et al. [6] used three types of fluorescence probes in their study of the binding surface on CaM that is created upon calcium binding to CaM . They found that the binding was not dependent on the charge of the hydrophobic probe, since the negatively charged probe (ANS) they used has its charged group attached directly to the aromatic ring and still binds to the protein. The same is true for TNS. Thus it seems unlikely that the negative charge on FMN *per se* would prohibit the binding.

The location of the TFP binding sites on calmodulin is not presently known. Gariépy and Hodges [27] have studied the binding of TFP to troponin C. They provide evidence for a TFP binding site in the region between amino acids 92–102 of troponin C. By homology, this would correspond to the region 82–92 of CaM . This region offers both hydrophobic (two phenylalanines) as well as negatively charged glutamic acid residues that could interact with the drug. The presence of the negatively charged glutamic acid side chains may provide an explanation for the stronger binding of positively charged TFP to metal-saturated CaM with respect to the other probes discussed.

The evidence presented above for the binding of hydrophobic compounds to CaM as deduced from ^{113}Cd NMR spectra does not necessarily imply that the biological activity of these drugs is mediated via CaM . The concentrations of CaM and drugs used in this work are in the millimolar range, thus also the drugs having a binding constant to CaM of $10^3 - 10^4 \text{ M}^{-1}$ cause observable effects in the ^{113}Cd NMR spectrum. Such high concentrations are unlikely to occur in a situation *in vivo*. The anti-hypertensive drug felodipine has been shown to bind to CaM [16], resulting in a conformational change of the protein. However, the physiologically inactive oxidized form of

felodipine gives qualitatively the same alterations in the ^{113}Cd NMR spectra as the active compound (see Table 1). Furthermore, little information is as yet available on the binding of drugs to the enzymes that are activated or deactivated by CaM . Thus caution has to be taken in the interpretation of the results with respect to the physiological relevance of the binding to CaM .

Although the concept that hydrophobic drugs can bind to CaM is now widely accepted (for review see [4]), Norman et al. [28] showed that there was no correlation between the clinical efficacy of drug isomers and their inhibitory action on a system of phosphodiesterase/ CaM . Instead a good correlation between the octanol/water partition coefficient of the drug and its inhibitory effect exists. Moreover, Roufogalis [29], studying four analogues of chlorpromazine, found that only one of them is physiologically active, yet all of them inhibit the CaM activation of $(\text{Mg}^{2+} + \text{Ca}^{2+})\text{-ATPase}$.

Binding of hydrophobic molecules to CaM is reported to be calcium-dependent [6, 7, 9, 19]. To extend these findings we studied the interaction of D600 with CaM by ^1H NMR as a function of the calcium content. It is interesting to note that several proton resonances from D600 split into two signals when D600 binds to $\text{CaM} \cdot \text{Cd}_4$. Is this due to slow exchange of the drug? The ^{113}Cd NMR results show that the conformational change in the protein due to D600 binding has a rate constant of about 2500 s^{-1} , indicating that the D600 exchange is at least that fast. Furthermore, the splitting of the proton signals always results in two equally intense lines independent of the D600, CaM or Ca^{2+} concentrations. Therefore, our interpretation of the splitting is that it is due to the fact that there is an asymmetric carbon in the D600 molecule and naturally the two enantiomers do not have to bind in the same way and with the same affinity to CaM . The splitting is only observable on the two methoxy signals from ring I, which is bound to the asymmetric carbon and on the *N*-methyl signal, which is four bonds away from the asymmetric center. The two methoxy signals from ring II do not show any splitting. It is therefore tempting to speculate that it is ring I that is bound to the protein and not ring II. The binding was observable as splitting of D600 signals already at $0.2 \text{ mol Ca}^{2+}/\text{mol CaM}$. This suggests that the presence of two bound Ca^{2+} ions/ CaM molecule is sufficient to create the binding domain of D600. At least two equivalents of Ca^{2+} is required for the binding of the fluorescence probe TNS to CaM according to the studies of Tanaka and Hidaka [7].

The alterations observed in both ^1H and ^{113}Cd NMR spectra of metal-ion-loaded CaM upon addition of either TFP or D600 are qualitatively the same. However, TFP is more strongly bound to CaM than D600. A competition study between TFP and D600 shows that TFP can replace D600 bound to $\text{CaM} \cdot \text{Ca}_4$. This indicates that at least one of the strong binding sites of TFP on CaM is common to TFP and D600.

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Paper VI

CHAPTER 3

The Interaction of Various Drugs with Calmodulin as Monitored by ^{113}Cd NMR

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I. INTRODUCTION

For a biochemist or a pharmacologist it may appear a very roundabout way to study the interaction of calmodulin (CaM) with drugs through the use of ^{113}Cd nuclear magnetic resonance (^{113}Cd NMR). However, we think it is fair to say that this approach provides information that would be difficult to obtain by other means and that it adds jointly with results from other methods to our understanding of the mode of drug binding, the location of drug-binding sites and the effects of drug binding on the structure and dynamics of the calmodulin molecule. NMR studies of the magnetic isotope ^{113}Cd are uniquely suited for the study of calcium-binding proteins (Forsén *et al.*, 1983). The ionic radii of Ca^{2+} and Cd^{2+} are very

similar and studies in our and other laboratories have indicated that Cd^{2+} is a reliable and convenient replacement for Ca^{2+} in a large number of calcium-binding proteins. In the case of calmodulin, ^1H -NMR studies indicate that the conformational changes induced by Cd^{2+} or Ca^{2+} binding to the four strongest cation-binding sites are virtually identical (Klevit, 1981; Forsén *et al.*, 1983; Thulin *et al.*, 1984). This is further substantiated by the findings that Cd^{2+} -saturated calmodulin (Cd_4CaM), just like Ca^{2+} -saturated calmodulin (Ca_4CaM), is able to activate CaM target proteins, for example, phosphodiesterase (Suzuki *et al.*, 1983).

In this chapter we first briefly describe the physical basis of the ^{113}Cd -NMR method and the experimental conditions used. Thereafter we summarize some recent studies of the interaction of different types of drugs with calmodulin and proteolytic fragments of this molecule; the latter have greatly aided the identification of the drug-binding sites on the CaM molecule.

II. EXPERIMENTAL DETAILS

In the study of calcium-binding proteins ^{43}Ca and ^{113}Cd NMR give complementary information about the protein-bound Ca^{2+} ions. For Ca^{2+} -binding sites having affinities (K_{Ca}) $< 10^6 - 10^7 \text{ M}^{-1}$ exchange rates of Ca^{2+} ions can be obtained from changes in the linewidth of the ^{43}Ca -NMR signal upon temperature variation (Andersson *et al.*, 1982; Drakenberg *et al.*, 1983). In the study of Ca^{2+} -binding sites having greater affinity, replacement of the Ca^{2+} ions by ^{113}Cd ions has proven useful (Forsén *et al.*, 1979, 1980; Andersson *et al.*, 1982). The ^{113}Cd resonances from protein-bound Cd^{2+} ions may, in contrast to ^{43}Ca resonances, be relatively narrow and well separated. The chemical shift range of ^{113}Cd NMR is large and dependent on the number and type of ligands bound to Cd^{2+} (Ellis, 1983). The ^{113}Cd -NMR resonances of calmodulin fall in the region expected for Cd^{2+} bound solely to oxygen ligands (Forsén *et al.*, 1980).

Due to the inherent low sensitivity of the ^{113}Cd -NMR technique (Harris, 1978), it is necessary to use rather high protein concentrations and isotopically enriched (96%) ^{113}Cd . In order to obtain a reasonable signal-to-noise ratio in the spectrum of a 1 mM protein solution (1 mM per ^{113}Cd equivalent, 3 ml) it has been necessary to accumulate for $\sim 8 \text{ h}$ (50,000 transients). A decrease of the protein concentration to 0.5 mM implies accumulation times of $\sim 30 \text{ h}$ if the signal-to-noise ratio is to be retained!

The ^{113}Cd -NMR spectra were obtained at 56.55 MHz. Typical acquisition parameters have been described elsewhere (Forsén *et al.*, 1979, 1980; Andersson *et al.*, 1983b).

III. BINDING OF CATIONS TO CALMODULIN

A. Addition of Cadmium Ions to Calmodulin

When cadmium ions are titrated into a CaM solution at high ionic strength (0.15 M KClO_4 , 23°C), two sharp resonances, A and B, at chemical shift positions -87.6 and -114.3 ppm, respectively, appear and increase in parallel, rather than sequentially, up to two added Cd^{2+} per CaM (Fig. 1a). An increase of the ratio of Cd^{2+} per CaM to 4:1 does not give rise to any detectable new signal. At higher ratios a broad resonance at

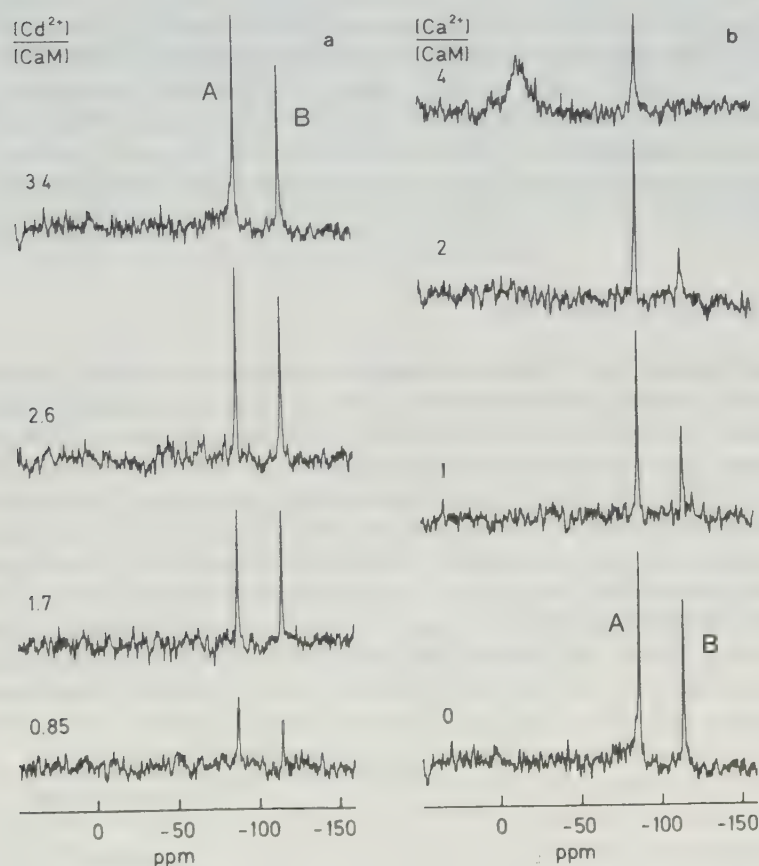


Fig. 1. Changes in ^{113}Cd -NMR spectra of CaM upon (a) addition of $^{113}\text{Cd}^{2+}$ and (b) addition of Ca^{2+} to the sample obtained after (a). The ratio of cation to CaM is indicated in the figure. Conditions used were: 14 mM CaM , 0.15 M KClO_4 , $\text{pH } 7.5$.

about -5 ppm appears in the ^{113}Cd -NMR spectra (not shown). These results are identical to those obtained at low ionic strength (Forsén *et al.*, 1980). Since the resonances A and B are observed to increase in parallel upon addition of $^{113}\text{Cd}^{2+}$ to apoCaM, this indicates that either the binding constants of the two high-affinity sites are almost identical or there is a strong positive cooperative binding. We have observed the same parallel increase for skeletal and cardiac troponin C (Forsén *et al.*, 1979; Teleman *et al.*, 1983), indicating that positive cooperativity is the more likely explanation, in line with the results of Crouch and Klee (1980). Also, ^1H -NMR studies provide evidence for cooperativity in binding of the first two calcium or cadmium ions (Teleman *et al.*, 1983). ^{113}Cd -NMR resonances from the third and fourth cation sites are not observable, presumably due to chemical exchange broadening caused by intermediate exchange between free and protein-bound cadmium ions (Forsén *et al.*, 1980). The broad resonance around -5 ppm arises from Cd^{2+} ions undergoing fast chemical exchanges between free ions and those weakly bound to the surface of the protein. Cd^{2+} ions also interact with Tris or anions like Cl^- , causing a change in the chemical shift and linewidth of the "free Cd^{2+} " resonance.

B. Metal Ion Competition

The addition of Ca^{2+} ions to a solution containing Cd_4CaM results in a decrease in the intensity of the two ^{113}Cd -NMR resonances, A and B, showing that Cd^{2+} and Ca^{2+} ions compete for the same sites (Fig. 1b) (Forsén *et al.*, 1980). Resonance B decreases more than resonance A, which shows that the ratio of the binding constants ($K_{\text{Ca}}/K_{\text{Cd}}$) is larger for site B than for site A. Furthermore, about half of the intensity of signal A remains even after the addition of four equivalents of Ca^{2+} ions, showing that $K_{\text{Ca}} \approx K_{\text{Cd}}$ for this site. It is invariably observed that signal A has a slightly higher intensity than signal B even when an excess of Cd^{2+} ions has been added. This difference may partly be explained as caused by a small amount of Ca^{2+} (≤ 0.2 eq) present in the apoprotein. Furthermore, T_1 measurements have indicated that the relaxation times of signals A and B are slightly different, 1.3 and 1.4 sec, respectively, for Cd_4CaM .

It is interesting that the same Ca^{2+} - Cd^{2+} competition experiments in the presence of trifluoperazine (TFP) have shown that the four signals observed under these conditions (Section IV,A) are affected differently, in analogy with the observation in the absence of drug the Cd^{2+} ions bound to the four sites are displayed by Ca^{2+} ions (Fig. 2). At a Ca^{2+} -to-protein ratio of 3 : 1, two of the four resonances have disappeared com-

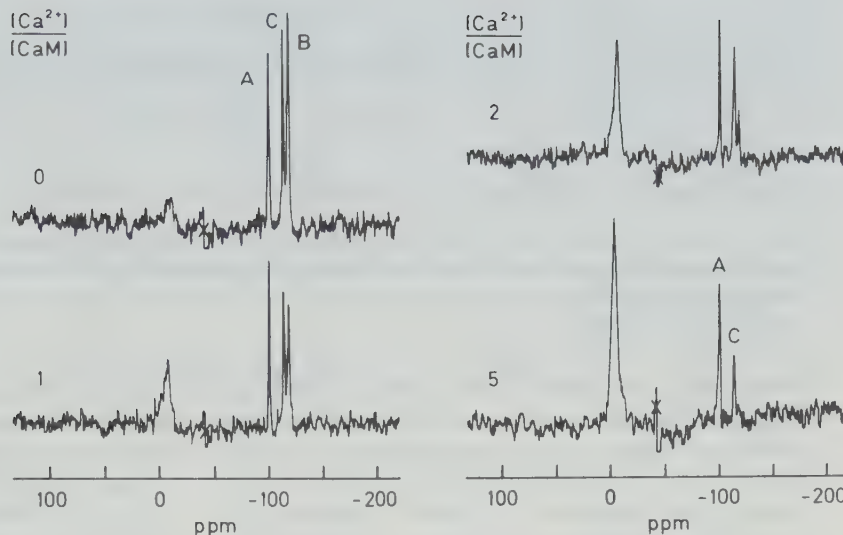


Fig. 2. Changes in the ^{113}Cd -NMR spectrum of a solution containing 1.4 mM Cd_4CaM and 4.2 mM TFP upon addition of Ca^{2+} , pH 7.0. The peak marked with a cross is an instrumental artifact due to an improperly balanced quadrature detector.

pletely, whereas one resonance remains essentially unaffected (Forsén *et al.*, 1980), that is, signal A in the drug-free spectrum. The two signals that have disappeared are signal B and one of the signals from the weak sites. When five equivalents of Ca^{2+} have been added one-third of the original intensity of signal C and two-thirds of signal A still remain (Fig. 2). This shows that the Ca^{2+} - Cd^{2+} selectivity of the two strong cation sites does not change with drug binding and that the two weaker cation sites, as well, have different $K_{\text{Ca}}/K_{\text{Cd}}$ ratios. This indicates the existence of two related pairs of cation-binding sites and, interestingly enough, a homology can be observed in the sequence of CaM between Ca^{2+} -binding domains I and III and between II and IV (Klee *et al.*, 1980).

C. Assignment of Strong and Weak Cation-Binding Sites

An important question for the understanding of the mechanism by which CaM regulates so many different metabolic processes is the order in which the four Ca^{2+} -binding sites of apoCaM (Fig. 3) are filled by Ca^{2+} . As regards this order many conflicting results have been reported (for a

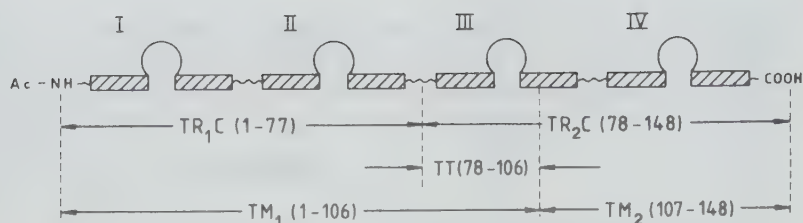


Fig. 3. Proteolytic fragments of CaM. The proposed structure of CaM is schematically shown, encompassing the four Ca^{2+} -binding domains, each consisting of a helix-loop-helix structure. Proteolytic fragmentation as induced by tryptic (TR), thrombin (TM), or a combination (TT) is indicated.

discussion, see Andersson *et al.*, 1983a; Thulin *et al.*, 1984). According to ultraviolet absorption changes of tyrosyl and phenylalanyl residues and NMR studies the four potential sites can be divided into two subclasses, two with lower and two with higher affinity for Ca^{2+} (Seamon, 1980; Klee *et al.*, 1980; Forsén *et al.*, 1980; Andersson *et al.*, 1982). For skeletal troponin C, a protein that is structurally related to CaM, the high-affinity Ca^{2+} - Mg^{2+} sites have been assigned to sites III and IV and the low-affinity Ca^{2+} sites to sites I and II from studies on proteolytic fragments (Leavis *et al.*, 1978). We have used similar proteolytic fragments of CaM in order to assign the strong and weak cation-binding sites.

Proteolytic cleavage with trypsin in the presence of Ca^{2+} occurs mainly at lysine-77 (Fig. 3), giving rise to two peptides of nearly equal size (Walsh *et al.*, 1977; Drabikowski *et al.*, 1977, 1982; Thulin *et al.*, 1984). The two peptides, TR_1C and TR_2C , retain most of the secondary structure they possess in the intact CaM molecule, as judged from circular dichroism (Drabikowski *et al.*, 1982) and ^1H NMR (Thulin *et al.*, 1984; Dalgarno *et al.*, 1984; Aulabaugh *et al.*, 1984). Moreover, the ^1H -NMR spectral changes induced upon addition of Ca^{2+} to the intact CaM are almost a superposition of the spectral changes observed upon addition of Ca^{2+} to the fragments (Thulin *et al.*, 1984). Parts (b) and (c) of Fig. 4 show the ^{113}Cd -NMR spectra of TR_1C and TR_2C , respectively. A summation of the two spectra gives a spectrum identical to that observed for the intact protein, part (a) in Fig. 4. We may thus conclude that resonances A and B in Cd_4CaM represent Cd^{2+} bound to cation-binding domains III and IV and that these sites constitute the high-affinity Cd^{2+} - and Ca^{2+} -binding sites (Andersson *et al.*, 1983a; Thulin *et al.*, 1984). Recent ^1H -NMR studies provide further evidence for this assignment (Ikura *et al.*, 1983; Thulin *et al.*, 1984; Aulabaugh *et al.*, 1984). Moreover, an increase in parallel of the ^{113}Cd -NMR signals of TR_2C , A' and B', is observed upon the successive addition of Cd^{2+} to the apofragment TR_2C just as for the intact protein (Section III,A), indicating cooperativity of cation binding. A com-

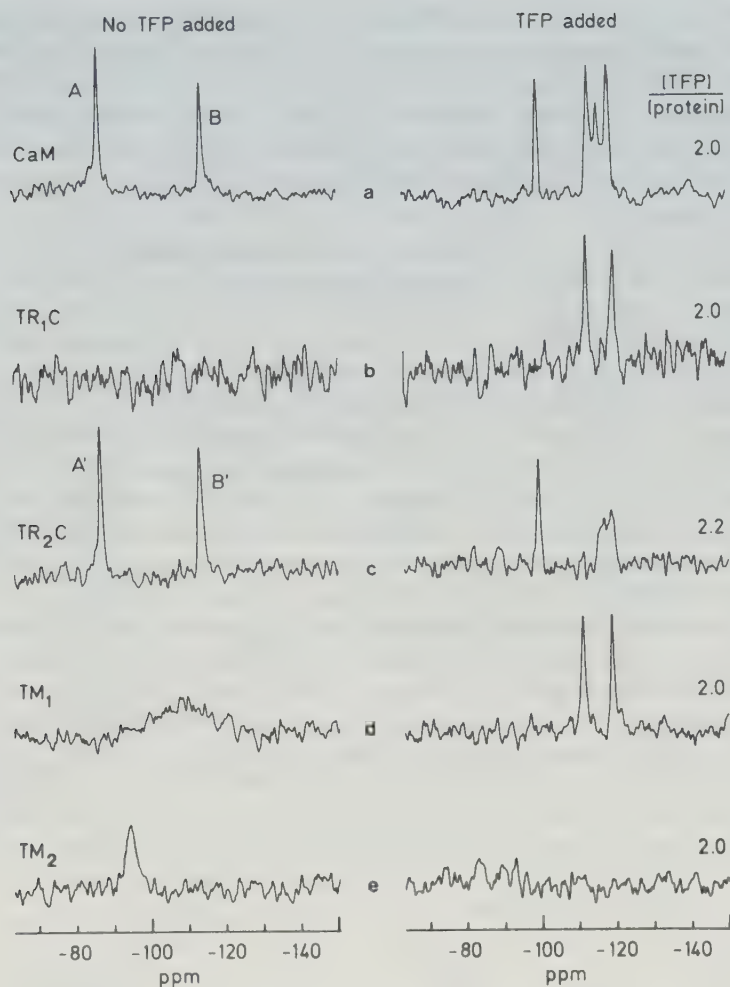


Fig. 4. ^{113}Cd -NMR spectra of CaM(a) and its fragments (b–e). The left column shows the spectra of Cd_4CaM and the Cd^{2+} -saturated fragments and the right column the resulting spectra after addition of TFP. The ratio of TFP to protein or peptide is indicated. Conditions used were as follows: (a) 1.4 mM Cd_4CaM , pH 7.5; (b) 0.97 mM $\text{Cd}_2\text{TR}_1\text{C}$, pH 7.5; (c) 0.82 mM $\text{Cd}_2\text{TR}_2\text{C}$, pH 7.5; (d) 0.92 mM TM_1 , 4.5 mM $^{113}\text{Cd}^{2+}$, pH 7.0; (e) 1.0 mM Cd_2TM_2 , pH 7.5.

petition experiment between Cd^{2+} and Ca^{2+} furthermore shows that Ca^{2+} preferentially displaces Cd^{2+} from the site corresponding to resonance B' exactly as in the intact protein (Andersson *et al.*, 1983a).

Proteolytic fragmentation with thrombin in the absence of Ca^{2+} will result in cleavage at arginine-106 (Fig. 3), giving rise to two fragments of

different size (Wall *et al.*, 1981). The ^{113}Cd -NMR spectra of the fragments TM_1 and TM_2 are shown in parts (d) and (e) of Fig. 4. The resonances observed are broad, indicating that their Cd^{2+} affinity is reduced compared to TR_2C (Andersson *et al.*, 1983a). This is consistent with the idea that a pair of related calcium-binding domains has a higher affinity for Ca^{2+} than one isolated domain (Vogel *et al.*, 1983b).

IV. INTERACTION OF VARIOUS DRUGS WITH CALMODULIN

A. General Effects

The ^{113}Cd -NMR spectrum of Cd_4CaM contains only two sharp resonances at -87.6 and -114.3 ppm, signals A and B, respectively (Section

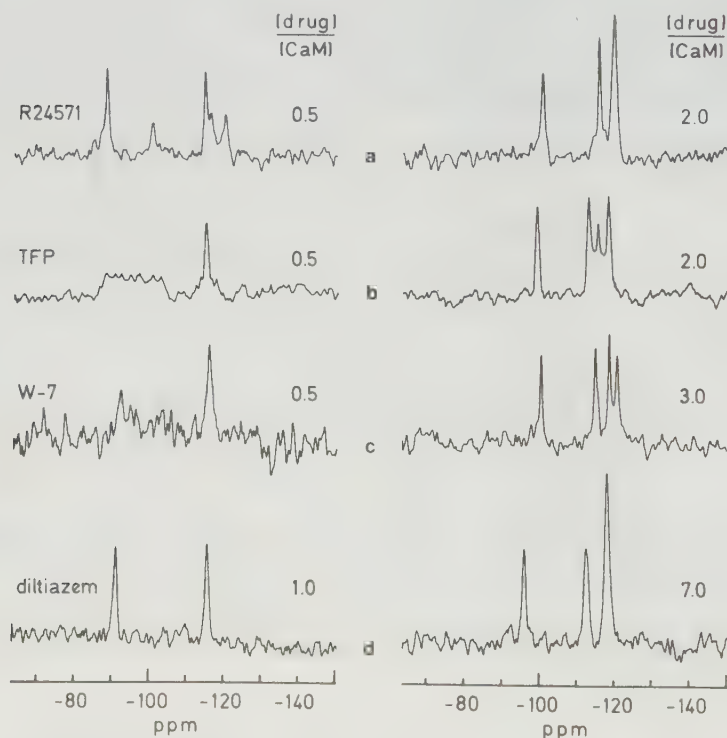


Fig. 5. The ^{113}Cd -NMR spectra of Cd_4CaM obtained after the addition of different drugs. The ratio of drug to CaM is indicated in the figure. (a) R24571, 1.2 mM Cd_4CaM , pH 7.1; (b) TFP, 1.4 mM Cd_4CaM , pH 7.0; (c) W-7, 0.62 mM Cd_4CaM , pH 7.1; (d) diltiazem, 0.77 mM Cd_4CaM , pH 7.5.

III,A). As observed hitherto two pronounced alterations in the ^{113}Cd -NMR spectra upon titration of CaM with drugs are common to most of the studied molecules that interact with CaM: (i) a pronounced upfield shift of resonance A and a small upfield shift of resonance B (Fig. 5 and 6) and (ii) appearance of a broad resonance that in most cases splits up into two narrow signals (Fig. 5) (Forsén *et al.*, 1980; Sudmeier *et al.*, 1980; Andersson *et al.*, 1983b). Since ^{113}Cd -NMR chemical shifts are extremely sensitive to small changes in protein structure (Cavé *et al.*, 1982; Ellis, 1983) the first finding implies that the environments of the two strongly bound cadmium ions change upon addition of drugs. The second finding reveals that the exchange rate for the cadmium ions bound to the two CaM binding sites that display intermediary exchange has been drastically reduced. The on-rate of cations to the four Ca^{2+} -binding sites is generally assumed to be diffusion limited (Bayley *et al.*, 1984), which means that the observed decreased exchange rate reflects an increase in binding constants of the cation (Forsén *et al.*, 1980). This conclusion is consistent with ^{43}Ca -NMR measurements (Andersson, 1981). Upon addition of TFP the NMR signal linewidth of the most rapidly exchanging calcium ion is drastically reduced. From the temperature dependence of the ^{43}Ca NMR linewidth in the presence of TFP and CaM it is possible to estimate the reduction of Ca^{2+} exchange to be at least 100-fold. Inagaki *et al.*, (1983) also have provided evidence for an increased binding constant of Ca^{2+} to CaM upon addition of drugs through equilibrium dialysis experiments.

Under certain conditions resonance A is observed to broaden. The maximum broadening yields an estimate of the lifetime, τ , of the drug- Cd_4CaM complex or, more correctly, the rate of the conformational change caused by drug binding. In the case of TFP, where a broadening of

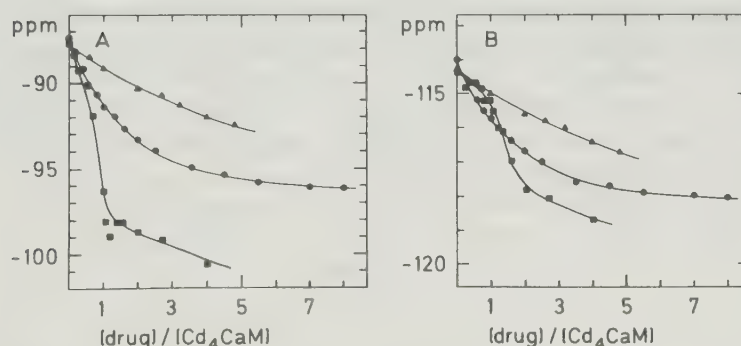


Fig. 6. The changes in chemical shifts of resonances A and B during the titrations of Cd_4CaM with TFP (■), diltiazem (●), and ethanol (▲).

signal A is observed between zero and one equivalents of TFP, the lifetime of the complex can be estimated to 10^{-3} sec (Forsén *et al.*, 1980). The dissociation constant of the TFP-CaM complex has been determined to be $\sim 1 \mu M$ (Levin and Weiss, 1977), indicating that the on-rate of TFP is close to diffusion limited.

The changes in the ^{113}Cd -NMR spectra following the addition of hydrophobic drugs can be divided into two groups: (i) marked effects caused by binding of the drug to about two specific sites and (ii) small upfield shifts of signals A and B caused by nonspecific drug binding. The two kinds of interactions are observed for TFP (Fig. 6). The change in chemical shift of signal A is nearly complete after the first added equivalent, and most of the change of signal B occurs upon addition of the second equivalent of TFP. This indicates two high-affinity TFP binding sites. Further addition of TFP causes only a small change in the chemical shifts of resonance A and B, indicating a weak nonspecific interaction. This is consistent with the findings from equilibrium dialysis experiments (Levin and Weiss, 1977) and ^{43}Ca -NMR studies (Andersson, 1981).

B. Specific Effects from Various Drugs

The hydrophobic drugs that have been shown to interact with calmodulin can be grouped together in various ways. An obvious grouping is in terms of their action, resulting in calmodulin antagonists (R24571, W-7, and TFP) and Ca^{2+} antagonists (D-600, diltiazem, and TNS). The calmodulin antagonists have dissociation constants of at most a few micromoles from the drug- Ca_4CaM complex and will bind sufficiently strongly to inhibit, for example, the calmodulin-induced activation of phosphodiesterase (Levin and Weiss, 1977; Hidaka *et al.*, 1980; Van Belle, 1981; Johnson and Wittenauer, 1983). The drug R24571 has some special effects on the ^{113}Cd -NMR spectrum due to its very low dissociation constant. The Ca^{2+} antagonists, however, have dissociation constants above $\sim 30 \mu M$ (Johnson and Wittenauer, 1983) and will not inhibit the calmodulin-induced activation of phosphodiesterase. A third group of hydrophobic compounds showed no specific binding.

When using ^{113}Cd NMR it is necessary, due to the inherent low sensitivity of the technique, to use concentrations of at least 1 mM. This means that all calmodulin and calcium antagonists will appear to bind very strongly in these experiments and little information regarding the strength of the binding will be gained in this way. However, we have observed that there is a correlation between the dissociation constant and the exchange rate of the drug molecules, which indicates that the binding is diffusion

limited. The conformational change in the protein caused by the binding of the drug should thus not be rate limiting.

The ^{113}Cd -NMR spectra of Cd_4CaM with less than saturating amounts of R24571 present show that the off-rate of the drug from its binding site(s) on the protein is slow ($k_{\text{off}} < 30/\text{sec}$), since two sets of ^{113}Cd resonances appear with two resonances from Cd_4CaM and four from R24571- Cd_4CaM (unpublished results from our laboratory). At saturating amounts of R24571 only the four resonances from R24571- Cd_4CaM are observed; in fact only three resonances can be resolved because two resonances accidentally coincide (Fig. 5).

The other calmodulin antagonists with dissociation constants in the micromolar range, TFP and W-7, show intermediate exchange behavior in their interaction with calmodulin, manifested in severe line broadening in the ^{113}Cd NMR spectra (Fig. 5). This line broadening, although troublesome because resonances sometimes disappear, adds another piece of information. When the ^{113}Cd chemical shifts of both Cd_4CaM and drug- Cd_4CaM are known the line broadening can be used to calculate the lifetimes of the various species. It has thus been found that the off-rates are $\sim 10^3/\text{sec}$ for both TFP (Forsén *et al.*, 1980) and W-7 (unpublished results from our laboratory). The dissociation constants for both drugs are reported to be $\sim 5 \mu\text{M}$ (Levin and Weiss, 1977; Hidaka *et al.*, 1980; Johnson and Wittenauer, 1983), therefore resulting in on-rates for the drugs of $\sim 2 \times 10^8/\text{M}/\text{sec}$. This should be compared to an upper limit for the on-rate for R24571 of $10^{10}/\text{M}/\text{sec}$ that can be derived from the ^{113}Cd -NMR results.

Also, felodipine, an antihypertensive agent, belongs, according to its dissociation constant from calmodulin (Johnson and Wittenauer, 1983; Johnson, 1983) and its inhibition of the heart calmodulin-induced activation of heart phosphodiesterase (Boström *et al.*, this volume) to the calmodulin antagonists. However, the ^{113}Cd -NMR results obtained for felodipine do not agree with those obtained for TFP and W-7. Felodipine undoubtedly binds to calmodulin both in its reduced, active form (Boström *et al.*, 1981) and its oxidized, inactive form (Andersson *et al.*, 1983b), since the ^{113}Cd -NMR spectrum changes with the addition of the drug. However, these changes are not easily interpreted and not even for an excess of felodipine are all four possible ^{113}Cd -NMR resonances observed. It is therefore clear that the mode of binding for felodipine is different from that of the other calmodulin antagonists.

It may be of some interest to discuss the TFP titration results in more detail. Initially, signal A broadens severely and shifts to lower frequency. A maximum broadening is observed for a TFP/CaM ratio of 0.5:1, indicating that this broadening is caused by the binding of TFP to *one single*

binding site on the protein. After the addition of one equivalent of TFP signal A has again become narrow and is not affected significantly by higher concentrations of TFP. Signal B, however, shifts only slightly to lower frequency for increasing additions of TFP up to a TFP/CaM ratio of 1:1. However, for TFP/CaM ratios from 1:1 to 2:1 signal B shifts ~ 3 ppm to lower frequency with no accompanying broadening, indicating an exchange rate for this process of 2000/sec or higher. This means that the off-rate for the second TFP molecule to be bound to the protein is higher than for the first one, in agreement with a diffusion-limited process. The second TFP molecule bound to the protein will also affect the two weaker metal ion-binding sites such that the exchange rates become low enough to allow us to observe sharp ^{113}Cd -NMR signals also for the cadmium ions bound to these sites.

As discussed in Section III,C, the two strong cation binding sites are situated in the C-terminal and the two weak sites in the N-terminal half of the protein. We will therefore assume that the most strongly bound TFP molecule is bound to the C-terminal half and the second to the N-terminal half. This would then imply that the conformational change induced by the binding of a TFP molecule to the N-terminal half of the protein also affects the conformation in the C-terminal half, since signal B shifts by ~ 3 ppm to lower frequency due to the binding of the second TFP molecule. ^{113}Cd -NMR studies of the TFP binding to the tryptic fragments TR_1C and TR_2C of CaM have also shown that there is one strong TFP binding site in each fragment (Section IV,D). It is here interesting to note that the ^{113}Cd chemical shifts for $\text{TFP}-\text{Cd}_2\text{TR}_2\text{C}$ are the same as for the two strong sites of $(\text{TFP})_2-\text{Cd}_4\text{CaM}$ and not for the ones in $\text{TFP}-\text{Cd}_4\text{CaM}$. This should then indicate that the shift difference of 3 ppm for signal B between $\text{TFP}-\text{Cd}_4\text{CaM}$ and $(\text{TFP})_2-\text{Cd}_4\text{CaM}$ or $\text{TFP}-\text{Cd}_2\text{TR}_2\text{C}$ originates in an interaction between the two halves of the CaM molecule that is not present in the latter two complexes; thus the second TFP molecule bound to Cd_4CaM appears to break an interaction between the two halves of the protein molecule.

The calcium antagonists with dissociation constants $>30 \mu\text{M}$, D-600 and diltiazem, and the fluorescence probe TNS all cause very much the same changes in the ^{113}Cd -NMR spectra when added to a Cd_4CaM solution (Andersson *et al.*, 1983b). All changes in the ^{113}Cd -NMR spectra occur in fast exchange, meaning that the chemical shifts will change continuously without any strong broadening of the resonances. The limiting chemical shifts obtained after the addition of an excess of drug are very similar for all three compounds and, furthermore, also similar to those induced by the calmodulin antagonists. We can therefore conclude that the calcium antagonists induce the same conformational changes as the

calmodulin antagonists. This indicates that the calcium antagonists fail to inhibit calmodulin activation because they bind too weakly to compete with the target enzyme even though they bind to the same sites as the calmodulin antagonists. Therefore we are led to believe that the interaction between the calcium antagonists and calmodulin has no physiological significance.

As shown in Fig. 6 even ethanol has an effect on the ^{113}Cd -NMR spectrum from Cd_4CaM , indicating that the nonspecific effect on the ^{113}Cd resonances observed for higher concentrations of drug is a general effect caused by any molecule that can interact with hydrophobic areas on the protein. Only for the water-soluble flavin dye FMN is there no observable effect on the ^{113}Cd -NMR spectrum from Cd_4CaM .

C. Drug-Binding Sites on Calmodulin

In Table I the chemical shifts are given of resonances in the ^{113}Cd -NMR spectra of drug-saturated Cd_4CaM . Three different drugs that have high affinity for CaM, R24571, W-7, and TFP, all give ^{113}Cd -NMR spectra that closely resemble each other. The major difference is that in the spectrum

Table I

Chemical Shifts for the Observed Signals in the ^{113}Cd -NMR Spectra of Cd^{2+} -Saturated Calmodulin when Drugs Are Added^a

Drug	Drug	δ (ppm ^b)			
	[Cd ₄ CaM]	signal A	signal C	signal D	signal B
None	0	-87.6	—	—	-114.3
R24571	2.0	-100.8	-115.9	-120.1	-120.1
W-7	3.0	-100.3	-114.5	-118.2	-120.3
TFP ^c	2.0	-99.1	-112.9	-115.2	-118.1
D-600	4.0	-96.1	-112.6	-116.3	-118.5
TNS	6.0	-98.0	-112.0	-119.9	-118.3
Diltiazem	7.0	-96.2	-112.7	-118.1	-118.1
Felodipine (red)	3.0	-91.4	—	—	-115.9
Felodipine (ox)	3.0	-94.5	~-110	~-110	-117.0
Ethanol	4.8 ^d	-92.5	~-110	~-110	-116.7
FMN	2.0	-88.0	—	—	-114.9

^a The final values are taken for the different titrations.

^b The ^{113}Cd -NMR chemical shifts are relative to a standard solution of $\text{Cd}(\text{ClO}_4)_2$ in water.

^c The chemical shifts of TFP are recalculated from an earlier report (Forsén *et al.*, 1980) by adding 0.6 ppm to the reference to obtain the same conditions as for the other drugs.

^d The value corresponds to the amount of ethanol that is added as solvent when 4.8 mol (D-600 or TNS)/mol CaM are added.

of the R24571-Cd₄CaM complex the two resonances most chemically shifted upfield are overlapping, while they are separated from each other with ~2 ppm in the case of TFP and W-7 complexes (Fig. 5). Since the chemical shifts in ¹¹³Cd NMR are known to be extremely sensitive to the protein conformation (Cavé *et al.*, 1982), the results strongly suggest that these three drug molecules induce the same overall change in the conformation of the protein. Very probably, these drugs bind to the same region of CaM. Other studies have been reported indicating that common binding site(s) exist (Levin and Weiss, 1979; Tanaka and Hidaka, 1980; La-Porte *et al.*, 1980; Johnson, 1983; Andersson *et al.*, 1983b).

At high drug-to-Cd₄CaM ratios, also, the less strongly bound drugs diltiazem, D-600, and TNS seem to induce similar changes as do the drugs with high affinity (Table I).

The only drug of the substances studied so far that gives qualitatively different chemical shift changes in the ¹¹³Cd-NMR spectrum is felodipine, both in the active reduced form and in the inactive oxidized form (Table I). The reason for this discrepancy is under further investigation in our laboratory.

D. Location of Trifluoperazine-Binding Sites on Calmodulin

Trifluoperazine is generally thought to inhibit the regulatory action of CaM on its target enzymes by competitive binding to a common binding domain on CaM (Levin and Weiss, 1979; Klee and Vanaman, 1982). In order to locate the two strong binding sites of TFP the ¹¹³Cd-NMR spectra of the tryptic fragments TR₁C and TR₂C (Section III,C), encompassing one-half of the protein each, were studied upon addition of TFP (Andersson *et al.*, 1983c; Thulin *et al.*, 1984). Two sharp resonances appear in the ¹¹³Cd-NMR spectrum of Cd²⁺-saturated TR₁C when TFP is added (Fig. 4). The decrease inferred from this in the cation exchange rate for the two weaker bound cations parallels that observed for Cd₄CaM upon addition of TFP (section IV,A). The two resonances are rather sharp at one equivalent of TFP, implying that one TFP molecule binds to TR₁C with high affinity. The two signals are observed to increase in parallel, indicating that the cation-binding sites are roughly equally affected by the binding of TFP.

Addition of TFP to Cd₂TR₂C causes resonances A' and B' to shift upfield, as observed for signals A and B in the case of the intact protein. A line broadening of signal A' is similarly observed between zero and one equivalent of TFP added. A splitting and line broadening of signal B' in

the later part of the titration of $\text{Cd}_2\text{TR}_2\text{C}$ with TFP is under further investigation in our laboratory.

When the ^{113}Cd -NMR spectra of TFP-saturated $\text{Cd}_2\text{TR}_1\text{C}$ and $\text{Cd}_2\text{TR}_2\text{C}$ are added, a spectrum closely resembling that of TRP-saturated Cd_2CaM is obtained (Fig. 4). Thus the strong binding sites of TFP on CaM seem to remain intact after the tryptic cleavage. One of the high-affinity sites is clearly located on the amino-terminal half and the other on the carboxy-terminal half of CaM (Andersson *et al.*, 1983c; Thulin *et al.*, 1984). This is consistent with results obtained from studies of interactions of the tryptic fragment with phenyl-Sepharose (Vogel *et al.*, 1983a).

In order to further locate the strong TFP-binding site on the carboxy-terminal half the ^{113}Cd -NMR spectra of the two thrombic fragments TM_1 and TM_2 (Fig. 3) were studied. Upon TFP addition to Cd_3TM_1 two sharp resonances appear (Fig. 4) with the same chemical shifts as observed with $\text{Cd}_2\text{TR}_1\text{C}$ (Thulin *et al.*, 1984). No change in the ^{113}Cd -NMR spectrum of Cd_1TM_2 can be observed upon addition of TFP. This is consistent with results obtained from the Ca^{2+} -dependent binding to phenyl-Sepharose (Vogel *et al.*, 1983a). Either TM_2 and TT (Fig. 3) have too low an affinity for Ca^{2+} to bind to the column in a Ca^{2+} -dependent manner or there is no binding site for phenothiazines in these fragments. In the latter case the phenothiazine binding site is to be found on the carboxy-terminal α -helix of cation-binding site III. Moreover, Krebs *et al.* (1984) found that in the presence of Ca^{2+} , TR_1C and TR_2C are labeled by 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine (TID), a hydrophobic photoreactive probe, whereas fragment TR_2E (107–148) is not. It is interesting the cyanogen bromide fragment CNBr1A – CNBr1B (77–124) binds in a Ca^{2+} -dependent manner to a column of fluphenazine-Sepharose (Head *et al.*, 1982), indicating that one hydrophobic site is located close to calcium ion domain III. It is thus unlikely that the TFP binding site on the carboxy-terminal half of CaM is solely located on the carboxy-terminal α -helix of calcium-binding domain IV, as has been suggested (Klevit *et al.*, 1981).

V. CONCLUSIONS AND FURTHER STUDIES

Studies using ^{113}Cd NMR have shown that the binding of drugs to CaM causes structural perturbations of the protein that influence all four cation binding sites. As a consequence the affinity of the protein for Cd^{2+} and Ca^{2+} is increased considerably. Preliminary results in our laboratory indicate that in the case of the $(\text{W-7})_2$ –CaM complex the binding constant of the two weaker binding Cd^{2+} ions has become comparable in strength to that of the high-affinity sites of CaM in the absence of drugs.

Judging from the changes observed in the ^{113}Cd -NMR spectrum upon the addition of excess drugs, all the drugs studied, with the exception of felodipine, cause very similar changes of the CaM structure. From studies of tryptic fragments of CaM (TR_1C and TR_2C) there is strong evidence for one drug-binding site on each fragment.

From the NMR spectroscopic point of view the distinction between CaM antagonists (W-7, TFP, and R24571) on the one hand and Ca^{2+} antagonists (D-600, diltiazem) on the other is merely one of strength of interaction with CaM, the former having a much stronger binding than the later. The CaM antagonists should consequently compete more effectively with the target proteins for CaM.

Further work is needed in order to obtain a complete assignment of the ^{113}Cd resonances seen in the drug- Cd_4CaM complexes. To this end studies of additional CaM fragments, for example, the cyanogen bromide fragment CNBr1A-CNBr1B (77-124) encompassing the Ca^{2+} binding domain III, might be highly informative.

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